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THE IMMUNOLOGY OF PARASITIC INFECTIONS

The Century Biological Series
Robert Wegner, Editor

THE IMMUNOLOGY OF PARASITIC INFECTIONS

BY
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TO

THEOBALD SMITH

THIS BOOK IS DEDICATED
IN RECOGNITION OF
HIS CLASSICAL INVESTIGATIONS ON
THE NATURE OF THE PIROPLASM INFECTIONS
IN PARTICULAR
AND OF HIS MANY CONTRIBUTIONS
TO OUR GENERAL KNOWLEDGE
OF PARASITOLOGY AND IMMUNOLOGY

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PREFACE

The present volume is not, in any sense, intended to be a treatise on immunology, but a compilation, and as far as possible, an evaluation of the mass of immunological work that has been done on infections with animal parasites. Besides stimulating interest in this field, I hope that the present work will hasten the incorporation of these experimental data into both parasitology and immunology, where, in spite of a growing interest during the past few years, it has been largely neglected. By achieving this, the parasitologist will undoubtedly gain new concepts for many of his experimental studies on infections, and the immunologist can obtain unique material for certain immunological questions. In making the last statement, I do not wish to intimate that the animal parasites are the best or even good material for general immunological studies where the use of purified compounds rather than living invaders or biological mixtures shows the greatest promise; but for the actual study of infection which must be made with the invading organism, many of the parasites, because of their accessibility and large size, offer unique material. Among certain protozoa, these characteristics make it possible to study the course of infections and the action of the host's resistance on the parasite in a direct manner which is impossible with the smaller bacterial invaders. Among the worms, they facilitate procuring large quantities of parasitic material. Finally, judging from past results, both in bacterial and parasitic immunity, a careful study of the immunological phases of parasitology will eventually yield many practical results—in particular, methods of diagnosis.

It is my experience that the zoölogist's concept of zoöparasitism differs somewhat in its viewpoint and in its use of certain terms from the concept of bacterial parasitism held by many bacteriologists and workers in medical fields, by whom most of the work in immunology has been done. Furthermore, the parasitologist is often unfamiliar with immunological terminology. To obviate these difficulties, a few of the fundamental concepts of zoöparasitism, infection, and immunity have been discussed in the introductory chapter.

The references in the bibliography have been given in full, with the idea that they may be of particular value to students interested

in this field. In some phases of the immunology of parasitic infections, notably in the cellular basis of immunity, in the production of alleged toxins and in natural immunity, it has been impossible to review all of the papers or even to list all of the numerous investigations. Instead examples have been selected and considered in detail, and, whenever possible, reviews have been referred to. In other phases, the bibliography has been made as complete as possible, but even here I am quite aware that there are many serious but unintentional omissions.

The frequent changes in the nomenclature of some of the parasites during recent years makes it extremely difficult for investigators, not primarily parasitologists, to recognize the various names of some of even the commoner parasites. To ensure consistency in the text, I have endeavored to use the generally recognized, valid name for each parasite. Then, to assist readers to identify such names, I have listed in an appendix their more important synonyms and ordinary hosts.

Particular aid in the preparation of this volume has been received from various associates. Of these, my sincerest gratitude is to my wife, who has constantly aided in the preparation of the entire manuscript, and to Professor Carrol G. Bull of Johns Hopkins University, who has read and criticized it. In addition, various colleagues have read those chapters related to their own specialties, among whom special mention should be made of Professor Paul R. Cannon and Dr. Frances A. Coventry of the University of Chicago. Finally, mention should be made of the technical aid received from Miss Anna B. Fisher, Miss Sara Goodloe, Miss Alice Gardner, and especially Miss Theodora Platt, who assisted in preparing the manuscript for publication.

The University of Chicago,
March, 1929.

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THE IMMUNOLOGY OF PARASITIC INFECTIONS

CHAPTER I

INTRODUCTORY CONCEPTS OF PARASITISM, INFECTION AND IMMUNITY REACTIONS

In attempting to define biological terms and concepts, one is confronted with the fact that most terms used in biology defy unequivocal definition because, in the first place, biological phenomena are not discrete entities, but form a continuous series in which one entity grades into the next; and, in the second place, the terms used in a rapidly advancing experimental science are tentative, and their meanings evolve with the accumulation of new data. There are, however, a few elementary concepts of parasitism and immunity which should be briefly outlined before considering the experimental work.

I. *Animal Parasites* *

The term *parasitism* is used by zoölogists in two different senses, in a general and in a restricted sense. Broadly speaking, any animal which lives, either temporarily or permanently, in, on, or with another living organism from which it obtains its nourishment is a *parasite,* while the organism which supplies the nourishment, and which is usually the larger and stronger, is the *host.* Such a concept, perforce, includes many forms not ordinarily considered parasites. Thus, many medical entomologists classify mosquitoes as parasites because the gradations among the various blood-sucking insects are so continuous that one cannot logically do otherwise, as evidenced by the following series given by Root (1924):

"(1) A wood mosquito (*Aëdes*), which will feed on blood when opportunity offers, but is entirely capable of completing its life cycle without it.

"(2) A malaria mosquito (*Anopheles*), which visits houses to obtain blood and is unable to develop eggs without it.

"(3) A yellow fever mosquito (*Stegomyia*), which not only visits houses for blood, but lives and breeds in and around houses, as well.

"(4) A bedbug, which lives and breeds in houses, visits human beings for blood, and shows degeneration of its wings.

* Interesting papers on parasitism among the protozoa, helminths, and insects have been contributed by Hegner (1924), Faust (1925), and Root (1924), respectively, and on the general subject of parasitism by Caullery (1922).

"(5) A flea, which breeds in houses (or in the nest of its host), has lost its wings, which visits the host for blood, and may remain on the host longer than is necessary for feeding alone.

"(6) An ordinary louse-fly (*Hippobosca,* for example), which normally lives on the body of its host and feeds on its blood, but retains well-developed wings and occasionally leaves the host for a flight through the air.

"(7) A wingless louse-fly (such as the 'sheep tick,' *Melophagus*), which always lives and feeds on the body of its host, just as a louse does."

Carried farther, parasitism grades into predatism, but a predacious animal generally kills and directly devours its prey, whereas a parasite lives at the expense of another without, even in those cases where it is lethal, immediately killing its host.

Parasitism, still in the broad sense, embraces every gradation of host-parasite relationship from cases in which the partners are mutually and equally beneficial to cases in which the parasite is pathogenic and even lethal to its host. Roughly, there are three types of association:

1. Symbiosis, in which both parties are benefited. An excellent example of this has been studied by Cleveland (1924). Termites live normally on cellulose, but they cannot digest it unless they harbor certain species of symbiotic flagellates. Deprived of these, they starve to death on pure cellulose, but can live if it is "predigested" outside of the body by certain microörganisms. In this case, the protozoa receive their habitation and nourishment; the termites, their mechanism for digesting their ordinary food.

2. Commensalism, in which the benefit is one-sided, that is, the parasite acquires its nourishment due to its association with the host, but the host is neither appreciably harmed nor benefited. *Endamœba coli* of man, which eats various bacteria and intestinal debris, probably removes nothing of value to man, and hence is a true commensal. Commensalism shades into the condition where the host is deprived of digested food which is of value and for which Minchin (1912) has coined the term *food-robbers.*

3. True parasitism. In the restricted sense, true parasites live at the expense of their hosts and are always of potential harm to them, either directly, by depriving them of essential material or destroying tissues, or indirectly, by damaging their hosts by the liberation of toxins or toxic materials.

Investigators working with the disease-producing parasites (all of which are necessarily true parasites) often overlook the fact that

it is the exception, not the rule, for a true parasite to be lethal to its host. Among the trypanosomes, for example, there are dozens of so-called non-pathogenic species to the few which produce disease in man and domesticated animals: and in the case of *Trypanosoma gambiense,* there is a growing mass of evidence that, while it is one of the most deadly parasites of man, it is naturally a parasite of certain wild game in which it does not produce observable symptoms and may, even, be no more harmful than the well-known *Trypanosoma lewisi* of rats. Indeed, these and numerous similar cases have induced many parasitologists to look upon cases of quickly lethal parasitism as examples of a recently associated relationship where the two are ill adapted to each other. In fact, it has often been pointed out that if the host and parasite cannot strike a balance, the parasite is at a disadvantage in the struggle for existence, since in killing its host, it also kills itself.* Practically nothing is known of the mechanism of this mutual adaptation, but in the case of *Trypanosoma lewisi* in the rat, as will be seen later, there is evidence that the host has acquired the power to form an antibody which eventually inhibits reproduction of the parasites; while in the lethal infections so far studied, the hosts do not develop this power.

Parasites are frequently divided into ectoparasites (ectozoa) and endoparasites (entozoa), according to whether they live on or within their hosts. Even this distinction is difficult to make at times. Consider such a form as the crablouse (*Phthirus pubis*), which is undoubtedly an ectoparasite, and yet, as will be seen later, may engender the production of a specific humoral response in the blood of the infected individual. Thus it initiates a chain of phenomena quite characteristic of true endoparasites.

The parasites of interest to medical and veterinary science belong to four phyla of the animal kingdom, the protozoa, the platyhelminthes (flatworms), the nemathelminthes (roundworms) and the arthropoda

* The ideas outlined here in regard to the activities of true parasites and their attempt to adapt themselves to their hosts so as not to kill them are well outlined in the popular and delightful presentation of van Beneden (1876) on *Animal Parasites and Messmates*—a book with which all students of parasitism should be familiar. According to him, "the parasite is he whose profession it is to live at the expense of his neighbor, and whose only employment consists in taking advantage of him, but prudently, so as not to endanger his life. He is a pauper who needs help, lest he should die on the public highway, but who practises the precept—not to kill the fowl in order to get the eggs. It is at once seen that he is essentially different from the messmate who is simply a companion at table. The beast of prey kills its victim in order to feed upon his flesh, the parasite does not kill; on the contrary he profits by all the advantages enjoyed by the host on whom he thrusts his presence."

(in particular the insects and arachnids). Many of these, among the protozoa and particularly among the roundworms, insects, and arachnids, are essentially similar to their free-living relatives with minor changes in habits or structures or both. Others, among the protozoa and particularly among the flatworms, exhibit profound structural changes, especially in the direction of increased reproductive activity.

II. *Course of Infections*

Once an entozoön has successfully invaded its host, there are certain characteristic phases of the resulting infection. Ordinarily, the terms used for these phases have been taken from clinical terminology dealing with symptoms. While this is sometimes confusing. the context usually makes it clear in which sense the term is used.

Incubation period is one of the most generally used terms. In the clinical sense, it refers to the time elapsing between the act of infection and the first appearance of symptoms. In the parasitological sense, it applies to the time between the act of infection and the appearance of the parasites in some specified organ or excretum of the body. Obviously, it is a very elastic term, and in some infections there may be several incubation periods, depending on the end point specified. Consider the infection of a dog with hookworm. Here, there are parasitological incubation periods terminated by the appearance of the worms in the skin, lungs, and intestine, and of the eggs in the feces. The objection might be raised that ordinarily there is only one incubation period, which is ended when some stage of the parasite can be recognized by laboratory diagnostic procedures, and that in the example just given, this would be only when the eggs appeared in the feces. This does not, however, sharply delimit the term. When an animal eats trichinous meat, a laboratory diagnosis may be made during the intestinal phase by fecal examination and demonstrating the adult worms, or during the later muscle phase by excising small pieces of muscle and finding the parasites. Thus, the parasitological incubation period has to be defined more or less for each infection. In general, however, among the protozoa, it is simply a period of unfettered reproduction during which the parasites are accumulating until they reach a number sufficient to be discovered by laboratory methods; among the worms, it is frequently the time of migration and growth of the larvæ to sexually mature adults.

Immunologically, the incubation period is of great importance, since it represents the first contact between host and entozoön, and

consequently the beginning of sensitizing and immunizing processes. In fact, during this period, immunological processes are generally well initiated, so that during the part of the infection actually under observation in the laboratory, the parasites are already living in an environment containing antibodies, and not in a normal animal. Von Pirquet and Schick (1903) have suggested that the clinical incubation period is roughly the time required for the body to become sensitized, allergic, or hypersensitive to the virus so that various materials liberated by the infecting organism, not toxic for the normal animal, become so in the infected and hence sensitized one. This mechanism of the production of symptoms will be discussed later.

Among the protozoa, once the parasites are established in a given site, the course of the infection depends on two factors: the reproductive activity and life-cycle of the parasite, and the defense of the host. In the blood protozoa, for example, there is often an acute increase in numbers (*acute rise*), which may progress until the death of the host, or which may be terminated by a sudden destruction of the parasites (sometimes aided by an inhibition of their reproduction). The periods of more or less sudden destruction have been designated *crises,* and as these always apply to number curves of infections, the present author generally calls them *number crises* to differentiate them from clinical terminology. Subsequently, the parasites often cannot be found for long periods in the blood, but may reappear. The periods of absence have been termed *latent periods* and the reappearances, *relapses*—again borrowing clinical terms.

Such concepts as acute rise, number crisis, latent period, and relapse do not apply in the ordinary sense to most helminth infections. Take, for example, a common infection of hookworm. Each adult worm represents a single infective larva acquired, ordinarily, from the ground. Hence, there can be no acute rise except in the sense that a large number of larvæ may gain entrance at about the same time, and therefore reach maturity at the same time. Furthermore, the death of the worms is a matter of accident or senescence and a recurrence is a matter of reinfection. Thus, true relapses can never occur. From these facts, it is evident that no hard-and-fast terminology will be adequate, and that the course of each helminth infection must be outlined separately.

In this connection it is well to note that recently Hegner (1926, 1926 b, and 1927) has proposed to retain the old terminology for the clinical periods and to adopt a different terminology for the parasitological periods of infection. He considers as the *patent period* the interval of the infection when "the parasites can be demonstrated by

the technique employed." The parasitological incubation period becomes the *prepatent period,* and the latent period the *subpatent period.* Admitting their appropriateness, these new terms will not be used in the present work because the older ones have been generally used for years in parasitological literature and are ordinarily clear from the context. Furthermore, the inclusion of the initial acute rise of parasites and all subsequent relapses under one term is confusing in infections with certain protozoa such as the trypanosomes, where there is evidence that the parasites of the acute rise and each relapse (relapse I, relapse II, etc.) are different in regard to their resistance to antibodies and their antigenic properties.

III. *Immunity*

I. ANTIGENS AND ANTIBODIES

Historically the science of immunity was concerned with the resistance of the body to disease. Soon, however, investigators in the field centered their attention on certain specific biological reactions that occurred when various foreign materials were introduced into the body. These foreign materials, termed *antigens,* may be infective organisms or derivatives of them, but may also be derived from a large number of other sources (viz., blood, purified egg-albumen, etc.). This has led to the building up of an entire science of serological reactions, all of which are probably related to the body's defense against disease, but many of which may play little or no direct part in such defense. Many of them have, however, supplied truly remarkably delicate reactions for the chemical study of proteins and accurate diagnostic methods for the laboratory study of disease.

These immunity reactions center around the fact that the parenteral introduction of antigens into the body is followed by the appearance in the blood of antibodies which react specifically with the antigens *in vitro.* Such antibodies are often termed *immune antibodies* to differentiate them from *natural antibodies,* which sometimes exist in the blood without immunization. Serum from the blood of an animal containing antibodies is generally known as an *antiserum.*

To act as an antigen, a substance must exist in a colloidal solution, must be foreign to the animal producing the antibody, and must penetrate beyond the epithelial surfaces which protect the body against foreign colloids (Wells, 1925). There is no question that

most soluble proteins are antigenic and there is considerable evidence that only proteins can be antigenic. (See Wells, 1928, and Branham, 1928.) Considerable confusion, however, has arisen because the term *antigen* has been used in two ways: (1) to designate those substances that stimulate the production of antibodies when introduced into animals, and (2) to designate those substances which react specifically with the antiserum outside of the animal body. Strictly, the term should always be limited to the first definition, i.e., substances which stimulate antibody production. As to the substances which react specifically with the antibody outside of the body, the following facts are interesting:

1. In many cases they are identical with the true antigens. Thus, if an animal be immunized with a highly purified protein and the *in vitro* reactions of its serum with solutions of the same protein be studied, undoubtedly the true antigen is the same as the specific reacting substance.

2. Zinsser and his associates found that they could isolate from certain bacteria non-protein substances which were not themselves antigenic, but which would react specifically in the test-tube with antiserums produced by immunizing with the whole bacterial cells. These substances they called *residue antigens*. A tremendous advance from the chemical side was made when Avery and Heidelberger isolated complex carbohydrates from pneumococci, which they termed *soluble specific substances*, and which behaved similarly. (For a review of this work, see Zinsser and Mueller, 1928.)

3. Similarly, lipoids react specifically with antiserums, but at present there is no clear-cut evidence that they can act as antigens. Landsteiner calls these substances *haptenes*.

In the present work, all of these materials which react specifically with antiserums *in vitro* have been grouped under the comprehensive term, *test antigens*.

There is a tendency to treat antibodies as if they were definitely known chemical complexes, when, as a matter of fact, they are known only as properties or manifestations of antiserum, and are postulated only in terms of what the antiserums do under certain conditions, just as enzymes are known by what they do rather than by what they are. Their chemical nature is entirely unknown, although they are generally precipitated by half saturation with ammonium sulphate in the globulin fraction of the serum—some in the pseudoglobulin, others in the euglobulin fraction—and are believed to be proteins.

2. IMMUNOLOGICAL REACTIONS

The immunological reactions which are particularly considered in the chapters that follow may be summarized briefly as follows:

1. *Precipitation,* in which the true antigen is termed the *precipitinogen* and the antibody the *precipitin.* The phenomenon is recognized chiefly in *in vitro* work, and consists in the formation of a precipitate upon the admixture of the test antigen and the antiserum.

2. *Agglutination,* in which the true antigen is termed the *agglutinogen* and the antibody *agglutinin.* This phenomenon has been studied chiefly in *in vitro* work, and involves the clumping of the test antigen (always a suspension, consisting of such materials as bacteria, protozoa, or blood-cells) upon the addition of the antiserum. As the clumps increase in size, they generally precipitate.

3. *Lysis,* in which the true antigen is termed the *lysinogen* and the antibody the *lysin.* In this reaction, which has been studied both *in vivo* and *in vitro,* a mixture of the test antigen (generally a suspension of cells, such as bacteria, blood-cells, or protozoa) with the antiserum results in a disintegration or lysis. This reaction is brought about by at least two components of the antiserum—one thermostabile, which is specific and arises as a result of immunization, the other thermolabile, which is non-specific and is present in the normal animal. If the antiserum is not comparatively fresh or if it is heated to 56° C. for twenty minutes (inactivated), it is non-reactive, but it can be reactivated by the addition of fresh normal serum (in laboratory work guinea-pig serum is generally used). The thermostabile component, which is the antibody, and which arises after immunization and is specific, is termed variously the *amboceptor, intermediary body,* or *sensitizer.* The thermolabile component, which is in normal serum and is non-specific, is termed *complement* or *alexin.* The test antigen can be sensitized first with the amboceptor so that if complement is later added, lysis occurs, whereas the complement will not combine either with the test antigen or the amboceptor alone. This fact is made use of in the following phenomenon.

4. *Complement fixation,* which is based on the fact that in *in vitro* experiments, complement will not unite with either amboceptor or test antigen until the test antigen has been sensitized, i.e., united with its specific amboceptor or antibody. Thus, if a test antigen and its specific antibody are placed in contact with complement, the three

combine and the complement is said to be "fixed," whereas if either the test antigen or amboceptor (antibody) is absent, the complement remains free in the solution. Whether the complement is free or "fixed" can be detected by adding to the test-tube sensitized red cells (red cells which have been in contact with inactivated hemolytic antiserum), whereupon lysis will not occur if the complement has been previously "fixed," but will occur if the complement is still free. Thus, the binding or fixing of complement becomes a delicate test for the presence or specificity of an antigen or an antibody.

5. *Anaphylaxis,* in which the antigen is termed the *anaphylactogen* or *sensibilisinogen* and the antibody the *anaphylactin* or *sensibilisin.* This reaction is exhibited in *in vivo* work usually, and is based upon the fact that after the antigen has been injected into an animal, a second dose after seven or more days may severely or fatally intoxicate the animal even when the antigen is entirely non-toxic in ordinary first doses. The intoxication can also be demonstrated *in vitro* in certain organs or tissues taken from the sensitized animal. The term *anaphylaxis* has been used so loosely by a number of writers that it is well to note the following criteria which Wells (1925) considers as necessary before a given reaction can be called anaphylaxis:

"1. The observed toxicity of the injected material must depend upon the sensitization of the animal; i.e., the substance must not produce similar symptoms in non-sensitized animals.

"2. The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being therefore the same for all antigens with the same test animal, but differing characteristically with each species of animal.

"3. It should be possible to demonstrate passive sensitization with the serum of sensitized animals.

"4. It should be possible to demonstrate typical reactions in the virgin guinea-pig uterus strip.

"5. It should be possible to demonstrate amelioration or prevention of the bronchial spasm in guinea-pigs by proper use of atropin and epinephrin. [Of the characteristics listed, this is probably the least dependable.]

"6. The possibility that the observed symptoms are caused by capillary thrombosis or embolism must be excluded.

"7. After recovery from anaphylactic shock there should be exhibited a condition of desensitization under proper conditions."

6. *Toxin-antitoxin reaction,* in which the antigen is the *toxin* and the antibody the *antitoxin.* This phenomenon consists in the fact that when the antigen is poisonous, the antibody can neutralize it.

Of necessity, the test for this neutralization must be made *in vivo*. Some investigators use the term *toxin* to include all toxic substances, especially those produced by disease-producing organisms. Most immunologists restrict the term to those poisons which stimulate the production of antitoxins within the body. Coca (1925) aptly defines it as an antigenic poison. This restricts the term to those true soluble toxins, such as the so-called exotoxins of bacteria (e.g., *B. diphtheriæ*), and plant toxins (e.g., ricin) and snake venoms, all of which stimulate the production of antitoxins. The so-called endotoxins of bacteria and various poisonous substances, like morphine, are excluded because they do not engender antitoxic substances.

7. *Phagocytosis and opsonins.* The general question of phagocytosis of invading organisms by wandering and fixed tissue-cells, as well as the general cellular response of animals, will be postponed to later chapters. Mention should be made, however, of the antibodies termed *opsonins* which are found to a certain extent in normal animals, but which are greatly increased by immunization. The action of opsonins can be readily demonstrated either *in vivo* or *in vitro*. If leucocytes washed free of serum are mixed with certain bacteria, some of them are phagocyted. If, however, the bacteria are first treated with a specific antiserum, many more are phagocyted. In other words, the opsonins have "sensitized" or prepared the bacteria so that the leucocytes will ingest them. Some immunologists (compare Wells, 1925, p. 236) who look upon phagocytosis and lysis as being fundamentally the same consider that a specific antibody sensitizes the invading parasite so that enzymatic lysis occurs in one case inside the cell, and in the other, outside.

8. *Inhibition of reproduction.* As a specific property of antiserum, this reaction has so far only been demonstrated *in vivo* for certain non-pathogenic trypanosomes. A mixture of the antigen (a suspension of protozoa) with the antiserum results, on injection into a normal animal, in an infection in which the organisms live a number of days, but never undergo any reproduction or cell-division. (See Chapter III.) It seems that, if possible at all, it will be extremely difficult to demonstrate this type of reaction in bacterial infections due to the small size of bacteria. It has to be demonstrated with organisms large enough so that one can see the effect on cell-division. A mere stopping of the rate of increase of the organisms is not sufficient evidence that reproduction is inhibited, because the organisms may be continuing to reproduce, but some lethal agent (lysins, opsonins) may be killing them off.

It should be again emphasized that the nature of antibodies is unknown and it is even a mistake to consider it proven that they represent definite *material additions* to the blood as a result of immunization. They may be nothing more than changes in already existing substances in the blood, as, for example, altered surface forces, etc. Actually, "antibodies" are simply altered reactivities of the serum after some immunizing process, and the type of antibody which is found depends on the particular set of conditions under which the serum is tested, viz., precipitation, agglutination, lysis, etc. Some immunologists believe that there are only two fundamental types of antibody response—the response to antigenic poisons (antitoxins) and the response to foreign proteins, whether poisonous or not (precipitins, agglutinins, complement fixation, etc.). In spite of the attractiveness of this hypothesis and in spite of much in its favor, it cannot be considered proven as yet.

The antibodies that have been considered are humoral properties of the host, but in addition there are many tissue and cellular responses to infection. As is well known, there was at one time a division of immunologists into the so-called humoral and cellular schools of immunity, and a long polemical argument arose as to whether immunity was mediated through humoral or cellular responses of the body. Fundamentally, there seems no real difference, because in the final analysis it is the cell itself which has to respond. If the response is limited to the cell, it is a purely cellular response, whereas if the cell initiates certain changes in the serum outside of the cell, it is a humoral response. The question of the part played by the cells in immunity, particularly by the reticulo-endothelial system, is reserved for Chapter V; and in the same chapter, the question of the probable origin of antibodies from these cells will be discussed.

3. HOST RESISTANCE

Various terms in immunology such as *immunity, resistance, susceptibility* of the host and *pathogenicity, invasiveness,* and *virulence* of the invading parasite are relative terms. Thus, if a parasite is virulent for a given host, that host necessarily exhibits a low degree of immunity or resistance to that parasite, or, to state it conversely, that host is markedly susceptible to that parasite. The relativeness of these terms is well brought out by Falk (1928), who defines virulence as follows:

"Virulence $\propto \frac{1}{\text{Resistance (or Immunity)}}$ i.e., *virulence varies*

reciprocally as resistance or immunity." It is, therefore, necessary to use these terms and let the reader obtain their meanings from the context rather than from exact definition.

Throughout the entozoa some species infect only a few hosts —are very specific—while others infect a large number. No species of parasite possesses a universal infectivity, and conversely, no host possesses a universal immunity. When the immunity is an inborn constitutional characteristic, it is called *natural immunity*. The converse condition is called *natural susceptibility*. Individuals, naturally susceptible, can often secure more or less immunity; this immunity is known as *acquired immunity*. Conversely, naturally immune individuals can sometimes be made susceptible, a condition known as *acquired susceptibility*. As will be emphasized later, natural and acquired immunity may depend upon different factors.

In the present volume, particular stress will be laid upon questions involving acquired immunity or resistance. An acquired immunity may be either active or passive.

Active immunity may be acquired in the following manners:

1. By the uninfluenced course of an infection with the virulent parasites.

2. By a milder infection with less virulent parasites (attenuated in various ways before infection).

3. By infection with virulent parasites and subsequent treatment with suitable drugs.

4. By treatment with dead or disintegrated parasites or products of such disintegration. This method obviates any danger of infection and has been extensively used experimentally with the trypanosomes. Under this heading is also included the use of autolysed parasites or bacteria.

5. By treatment with products of the invading organism. The best example of this is seen where a true exotoxin, produced by the invader, is used to incite the formation of antitoxin. Thus, a horse can be immunized by successive subcutaneous injections of diphtheria toxin so that its blood contains antitoxin. A toxin detoxicated by heating (toxoid) or treating with formaldehyde (anatoxin) will still incite antitoxin formation.

6. Sometimes an acquired immunity towards one organism can be produced by immunizing with an entirely different organism.

Passive immunity differs from active immunity in that the protective substances are not formed by the animal, itself, but are conferred on it by the injection of protective substances from another

animal. Passive immunity is more quickly effective than active immunity, but does not last as long.

An acquired immunity is frequently produced by a combination of the methods for inducing active and passive immunity. For example, highly protective serum is combined with the active toxin in immunization against diphtheria (the toxin-antitoxin treatment).

IV. *The Environment of Parasitic as Compared with Free-living Animals*

All animals, to survive, must become so adapted to their environment that the individual and the race may be maintained. This is just as true of a parasite as of a free-living animal, the only difference being that the environment of a parasite is more concerned with other living organisms, for, at least during a part of its life-history, its environment *is* another living organism. This similarity has been emphasized by several recent writers. Thus, Hegner (1926) in discussing the homologies and analogies between free-living and parasitic protozoa, makes a comparison between the natural resistance of a host against a parasite and the adverse situations which a free-living protozoön may find in populating a pool of water; between acquired resistance of a host, and the unfavorable accumulation of secretions and excretions in the environment of a free-living form; between latency and relapse of infections, and variations in the census of free-living forms; and between host-parasite specificity, and the favorable and unfavorable conditions encountered by free-living species. Similarly, Hartman (1927) emphasizes that host-parasite relationships have a chemico-physical basis, and states that "probably the same chemical or physical agent would produce the same effect if it came from a non-living source as from the living one."

Such comparisons as these fail to emphasize one essential point. There is no question that various factors of host resistance, antibodies, cellular reactions, non-specific factors, etc., constitute the environment of the parasite and that these are recognizedly on a chemico-physical basis, * but the peculiar thing is that the adverse environmental conditions met by the parasite are so highly *specific*. This does not mean that all factors of host resistance show this

* Compare, for example, Wells (1925): "Immunological reactions, the processes by which the living organism defends itself against the chemical attacks of its enemies and so is able to exist in an environment seething with such enemies, are chemical reactions."

specificity, but the ones ordinarily classed as immunity reactions do have this characteristic in a high degree. Furthermore, these highly specific defense mechanisms of the host are formed, as far as is known, only in a living organism.

CHAPTER II

SEROLOGICAL REACTIONS USED IN DIAGNOSIS: COMPLEMENT FIXATION, AGGLUTINATION, PRECIPITIN REACTION AND MISCELLANEOUS TESTS

I. *Introduction*

By far the greatest part of the literature on the immunology of parasites deals with the test-tube serological reactions considered in this chapter, i.e., complement fixation, agglutination, precipitation, and miscellaneous serum tests. Although the three specific serological tests are of great fundamental interest in showing that the body responds to infection with parasites by the formation of specific antibodies, the majority of the work on them has been undertaken with the hope that eventually exact and practical methods of diagnosis could be perfected. This hope has been realized in only a few cases: in hydatid disease of man, in dourine of horses, and to a less extent in schistosome infections. As regards the miscellaneous serum tests, the work has been so exclusively directed toward their diagnostic value that their relation to immunological processes is as yet mere conjecture. One of the most successful of these tests, so far, has been devised for kala-azar.

The reader will find in this chapter many conflicting results and, at times, insufficient data to warrant satisfactory conclusions. The discrepancies have been included because serological reactions are so dependent on exact controlled experimental conditions that positive results by one investigator and negative results by another trying to repeat his findings may simply mean that the first worker unwittingly controlled certain necessary conditions which, as it happened, the second investigator did not. Such a condition of affairs may stimulate others to continue the study until the variable factors are consciously controlled. The sporadic papers in isolated fields have been included to show the scarcity of known facts or to indicate the trend to be anticipated for further work.

In presenting the data, I have been confronted with the dilemma that technique, which is one of the most essential problems of serological work, varies with almost every individual. This is preëm-

inently so in the formative stages of any test. Hence, it has been impossible to give the protocols for even the more important investigations. As a compromise, this introduction gives a standardized procedure, in brief résumé, of the more important methods (including the necessary controls) for each of the three serological tests; and the rest of this chapter is devoted to specific results, with especial reference to the salient features in the preparation of suitable test antigens. In addition, I have included pronouncedly different procedures, descriptions of the miscellaneous tests, and, in those diseases where a test has been sufficiently standardized to be extensively used, details of one of the techniques.

I. COMPLEMENT FIXATION

1. *Specific complement fixation.* The original work of Bordet and Gengou (1901) was not only the basis for all of the work on specific complement fixation, but also led to the *non-specific* complement fixation test for syphilis (referred to frequently as the Wassermann test). The Bordet-Gengou reaction or specific complement fixation reaction is based on the facts (1) that complement (alexin) is non-specific, whereas each antigen reacts with a specific antibody (amboceptor or sensitizer); and (2) that complement will not combine with either antibody or antigen alone, but only with the antibody-antigen complex (sensitized antigen). Since, therefore, an antibody will combine only with its specific antigen (or closely related antigens showing a group reaction), the binding or fixing of complement is proof of the presence of an antibody and its specific antigen. Accordingly, if a known test antigen is in the system, the fixing of complement becomes a delicate test for the presence of the specific antibody; or if a known antibody is in the system, the fixing of complement becomes a test for the specific test antigen. The fixation of complement is not usually apparent, however, until a known antibody-antigen complex is added which will act as an indicator of the presence or absence of free complement. Sensitized red blood-cells have been chosen as the standard. When these are added, if hemolysis does not occur, complement has been fixed, i.e., a specific test antigen and antibody were present; whereas if hemolysis occurs, complement was free, i.e., either the specific antigen or antibody was absent.

There are almost as many modifications in the technique of the complement fixation reaction as there are laboratories. Furthermore, various Wassermann techniques have been modified to suit the needs

of different specific fixation requirements. An excellent outline of the best of these may be found in Kolmer (1923 and 1928). In the following paragraphs, only the essential steps of one method will be briefly indicated to insure clearness in the later discussions.

THE REAGENTS AND THEIR PREPARATION

1. The equipment necessary, such as capillary pipettes, 1-c.c. pipettes graduated in hundredths to the tip, 5-c.c. and 10-c.c. pipettes graduated in tenths, tubes for the tests (12 × 100 mm.), diluting test-tubes and Erlenmeyer flasks (or large centrifuge tubes), syringes for drawing blood, etc., must be thoroughly cleaned and sterilized. Proper equipment is essential for success.

2. Sterile 0.85 per cent NaCl is used for all dilutions and washings unless otherwise noted.

3. The test antigen is prepared from isolated parasites or from heavily infected organs by diverse techniques, involving the use of fresh or dried material, extracted with lipoidal solvents (in various combinations in which either the soluble or insoluble portion may be used), with aqueous solvents, or with both. To be efficient, it must possess a high antigenic power (that is, a good combining power with its specific antibody) and a low anti-complementary power (that is, materials used as test antigens may inhibit the action of complement by themselves, thereby indicating a false fixing of complement).

4. The complement is preferably obtained by bleeding guinea-pigs aseptically from the heart, collecting the serum and storing it on ice. As a rule, the serum may be safely used for two days, but in any case its potency has to be checked by preliminary titrations.

5. For testing the presence of complement in the fixation test, most investigators use anti-sheep hemolytic serum from immunized rabbits and washed sheep red blood-cells. In the presence of complement, this antiserum has the property of lysing the red cells so that the cells are mere shells and the escaped hemoglobin colors the diluent a clear pink or red. For the best results, it must have a high titer (1 : 5,000). It can either be bought from a standard laboratory supply house or be produced in any of a number of ways. According to Stowell and Beattie (1925), the highest average titer of antiserum is produced by three daily intravenous injections of 1, 2, and 2 c.c. of 50 per cent (in terms of whole blood) washed sheep cells, a five-day interval, and then a similar series of injections into each of six rabbits (a number of rabbits is needed because

the titer of their serum varies considerably); the rabbits are then bled on the second day after the last injection and their serums are separated and preserved with glycerin (equal parts) on ice. (For titration, see Table 1.)

6. The cells are obtained by bleeding a sheep from the jugular vein into a bleeding bottle containing either heparin or sodium citrate, and subsequently washing three times with an excess of saline. They are then diluted by a carefully standardized procedure, since the suspension must be fairly constant from day to day. Thus, the cells may be pipetted into a graduated centrifuge tube, centrifuged at 2,000 r.p.m. for ten minutes, and diluted to the desired amount in an Erlenmeyer flask. (They may be stored on ice and used until they show signs of hemolysis.) Since the blood-cells and hemolytic antiserum have the ability to combine, without any change taking place until complement is added, they are often mixed in the desired dilutions (as such they are called *sensitized cells*) and added to the test-tube in that form.

It has been found that any one of the reagents used in the test may by itself have the accidental capacity either to inhibit the action of complement on the sensitized cells or to hemolyze the cells without hemolytic serum or complement. All of these possibilities are ruled out by setting up control tubes containing the separate reagents with the sensitized or unsensitized cells, which must either hemolyze or not according to the constituents of the tube. Should they react counter to expectation, fresh material must be obtained. Thus, in Table 1, tube 17, by exhibiting no hemolysis, shows that the antiserum is not hemolytic by itself. Tubes 18 and 19 are likewise controls of the guinea-pig serum and saline, respectively.

Furthermore, there is an optimum proportion for facilitating the reaction between the various factors. As the strength of the experimental antiserum and (until standardized) of the test antigen is unknown, it is necessary to have a very definite balance of complement, hemolytic antiserum, and sheep cells. For this reason, a standard has been arbitrarily accepted by most investigators and is as follows: The titer of the hemolytic serum is the smallest amount of undiluted serum necessary to hemolyze completely 1 c.c. of a 5 per cent suspension of corpuscles (one unit) in the presence of two units of complement. Two units of complement, strictly defined, is 0.08 c.c. of pooled normal guinea-pig serum, but many investigators, following the earlier workers, use 0.1 c.c. In some techniques, fractional units are frequently employed for economy of materials. In fact, in the titrations following, which are used by Professor C. G.

Bull of Johns Hopkins University and by us in this laboratory, fifth units are used throughout. Thus, 0.5 c.c. of 2 per cent corpuscles is one fifth unit, and 0.2 c.c. of 10 per cent guinea-pig serum (complement) is one fifth of the older arbitrary dose of 0.1 c.c. of undiluted serum.

In making the dilutions and in pipetting the amounts into the various test-tubes, it is obvious that great care has to be exercised. Parenthetically, it may be remarked that, in immunology, dilutions are generally expressed as one part of the reagent in so many total volumes: thus, a dilution of 1:4 signifies *one* part of the reagent and *three* parts of the diluent (one in four, i.e., a 25 per cent solution). Deviations from this procedure occur in this volume only in reprinting the tables of certain workers who indicate dilutions as one part of the reagent to so many volumes of the diluent.

PRELIMINARY TITRATIONS

Protocols for the preliminary titrations are given in Tables 1-3. In these protocols, all amounts are in cubic centimeters, each test-tube having a total of 2 c.c. In order to facilitate discussion, hypothetical results are indicated.

TABLE 1

PROTOCOL FOR THE TITRATION OF ANTI-SHEEP SENSITIZER

Tube	*Sensitizer*		*Saline: 0.85%*	*Complement (from 3 g.ps.) 1: 10*	*Sheep cells 2%*		*Possible results*
	Dilution	*Amount*					
		c.c.	c.c.	c.c.	c.c.		
1	1:100	0.8	0.5	0.2	0.5		Hemolysis
2		0.6	0.7	0.2	0.5		Hemolysis
3		0.4	0.9	0.2	0.5		Hemolysis
4		0.2	1.1	0.2	0.5		Hemolysis
5	1:1000	1.0	0.3	0.2	0.5		Hemolysis
6		0.8	0.5	0.2	0.5		Hemolysis
7		0.6	0.7	0.2	0.5		Hemolysis
8		0.5	0.8	0.2	0.5		Hemolysis
9		0.4	0.9	0.2	0.5		Hemolysis
10		0.3	1.0	0.2	0.5		Hemolysis
11		0.2	1.1	0.2	0.5		Hemolysis
12	1:10000	1.0	0.3	0.2	0.5		Hemolysis
13		0.8	0.5	0.2	0.5		Hemolysis
14		0.6	0.7	0.2	0.5		Hemolysis
15		0.5	0.8	0.2	0.5		Hemolysis
16		0.4	0.9	0.2	0.5		No hemolysis
17 C*	1:100	0.8	0.7	0	0.5		No hemolysis
18 C		0	1.3	0.2	0.5		No hemolysis
19 C		0	1.5	0	0.5		No hemolysis

Mix: Incubate at 37° C. 1 hour

* C = Control.

Table 1 gives a protocol for the titration of the anti-sheep rabbit serum. Inasmuch as this is an important basic titration, the complement should be in standard units as far as possible, and hence, should consist of pooled serum from a number of healthy normal guinea-pigs. According to the possible result, complete hemolysis occurs through tube 15, which contains 0.5 of 1 : 10,000, and, therefore, the titer is 1 : 20,000 in the fractional units or 1 : 4,000 in the standard units. Later, in using sensitized cells, it is necessary to have cells and sensitizer in such a dilution that each 0.5 c.c. contains 2 per cent cells and two units of sensitizer. This is easily done by mixing equal quantities of 4 per cent washed sheep cells and a dilution of sensitizer so that two units are contained in 0.25 c.c., which in the example above would be 1 : 2,500—in other words, an equal mixture of double-strength reagents. A titration for a given 1 : 10 dilution of the glycerinated antiserum will hold for about a week.

TABLE 2

PROTOCOL FOR THE TITRATION OF COMPLEMENT

Tube	*Complement 1: 10*	*Saline*	*Sensitized cells*		*Possible results*
	c.c.	c.c.	c.c.	Mix : Incubate at 37° C. 1 hour	
1	0.3	1.2	0.5		Hemolysis
2	0.2	1.3	0.5		Hemolysis
3	0.15	1.35	0.5		Hemolysis
4	0.1	1.4	0.5		Hemolysis
5	0.05	1.45	0.5		No or partial hemolysis
6 Control	0	1.5	0.5		No hemolysis

If the serums from a large number of healthy guinea-pigs are pooled, the unit of complement can be taken as a definite amount (in fifth units, 0.2 c.c. of a 1 : 10 dilution). When complement is obtained from one or two animals, it is necessary to rule out individual variations by a preliminary titration as given in Table 2. In the example given, since complete hemolysis occurs through tube 4, this is the unit of complement. In the actual fixation tests, two units are used or, according to the results in Table 3, 0.2 c.c. of 1 : 10. (In the standard system this would be 1.0 c.c. of 1 : 10). A much lower dilution is not considered desirable. This titration has to be repeated for every batch of serum, or at least every other day when tests are run regularly.

The next step before performing the actual fixation test is to ascertain to what extent the test antigen will non-specifically fix

TABLE 3

PROTOCOL FOR THE ANTICOMPLEMENTARY TITRATION OF ANTIGEN

Tube	Antigen		Saline	Complement 1:10		Sensitized cells		Possible results
	Dilution	Amount						
		c.c.	c.c.	c.c.		c.c.		
1	undil.	0.3	1.0	0.2	Mix: Overnight in icebox or 1 hr. in water bath	0.5	Mix: Incubate at 37° C. 1 hour	No hemolysis
2		0.2	1.1	0.2		0.5		No hemolysis
3		0.1	1.2	0.2		0.5		No hemolysis
4	1:20	1.0	0.3	0.2		0.5		No hemolysis
5		0.8	0.5	0.2		0.5		No hemolysis
6		0.6	0.7	0.2		0.5		Partial
7		0.4	0.9	0.2		0.5		Hemolysis
8		0.2	1.1	0.2		0.5		Hemolysis
9		0.1	1.2	0.2		0.5		Hemolysis
10 C *	undil.	0.3	1.2	0		0.5		No hemolysis

* C = Control.

complement. A protocol for this titration is given in Table 3. In the example given, since partial hemolysis occurs in tube 6 (0.6 c.c. of 1:20), this is the anti-complementary dose, and half of it can be considered a safe dilution for the actual test. Sometimes, an experimental test antigen may be hemolytic. In that event (e.g., if control tube 10 showed hemolysis), the above protocol should be repeated, leaving out the complement altogether. The subsequent dilution for the test would have to be higher than the one that caused hemolysis of the cells and higher than the anti-complementary dilution. These titrations, once satisfactorily performed, will be sufficient for any particular batch of antigen.

THE COMPLEMENT FIXATION TEST

When the preliminary titrations are completed, the actual complement fixation test may be performed according to the protocol given in Table 4. In the example given, since no hemolysis occurs in tubes 1-4, the complement was previously fixed by the experimental immune serum and test antigen (positive result), whereas, had hemolysis occurred in all tubes, it could have been concluded that either immune serum or its specific antigen was absent (negative result).

2. *The Wassermann reaction.* After Bordet and Gengou's work on specific complement fixation, Wassermann, Neisser, and Bruck (1906) used what they considered to be the same reaction for the diagnosis of syphilis. Later workers demonstrated, however, that

TABLE 4

PROTOCOL FOR THE COMPLEMENT FIXATION TEST

(The dilutions here are in accordance with the preliminary tests.)

Tube	Immune serum		Saline	Antigen 1:20	Complement 1:10		Sensitized cells		Possible results
	Dilution	Amount							
		c.c.	c.c.	c.c.	c.c.		c.c.		
1	1:4	1.0	0	0.3	0.2	Mix: Over night in ice-box or 1 hr. at 37° C.	0.5	Mix: 37° C. 1 hour	No hemolysis
2	1:8	1.0	0	0.3	0.2		0.5		No hemolysis
3	1:16	1.0	0	0.3	0.2		0.5		No hemolysis
4	1:32	1.0	0	0.3	0.2		0.5		No hemolysis
5	1:64	1.0	0	0.3	0.2		0.5		Partial
6	1:128	1.0	0	0.3	0.2		0.5		Hemolysis
7	1:256	1.0	0	0.3	0.2		0.5		Hemolysis
8	1:512	1.0	0	0.3	0.2		0.5		Hemolysis
9 C*	1:4	1.0	0.3	0	0.2		0.5		Hemolysis
10 C	1:8	1.0	0.3	0	0.2		0.5		Hemolysis
11 C		0	1.3	0	0.2		0.5		Hemolysis

* C = Control.

not only was it unnecessary to prepare test antigens from material containing the organism of syphilis, but that even better results could be obtained with test antigens consisting of extracts of *normal* tissues. With such a non-specific test antigen, it would seem likely, and has been frequently reported, that the Wassermann complement fixation test for syphilis might give positive results in other diseases, but as the test has become perfected, the truly remarkable thing is that more and more of these pseudo-positive reactions with other diseases have been ruled out. At the present time, therefore, a positive Wassermann test is very strong presumptive evidence of syphilis or the closely related spirochete infection, yaws. Gilbert (1928) sums up the present idea of the specificity of the Wassermann reaction as follows: "There are reports of definite fixation occurring with serum from cases of pregnancy and from patients suffering from various diseases including scarlet fever, pellagra, diabetes, tuberculosis, leprosy, malaria, relapsing fever, and yaws. As regards yaws, which is due to *Treponema pertenue,* an organism almost indistinguishable from *Treponema pallidum,* the literature indicates that a reaction can be expected with equal regularity as in the case of syphilis. There seems to be an indication also that in rare instances reactions may be secured with blood from cases of malaria, leprosy, and tuberculosis, even when syphilis is not present, but there is little evidence that in the other conditions mentioned there is any likelihood of reactions occurring when syphilis can be ruled out with reasonable

certainty. It is not considered desirable to secure specimens of blood when the patient has a fever or is under the influence of alcohol or an anesthetic." Similarly, Kolmer (1927), in speaking of his own carefully standardized Wassermann technique, states that positives only occur in syphilis and yaws and never in the host of other diseases which are supposed by various investigators to yield false positives. He believes that whenever these false positives do occur, a technical error is responsible. Inasmuch as this test with its normal tissue test antigen is so unquestionably specific for syphilis, the large amount of work dealing with attempts to apply it to the parasitic infections does not properly fall within the scope of the present book.

Although a positive Wassermann test is rarely obtained in diseases other than syphilis and yaws, the specific test antigens used in complement fixation tests for various other diseases sometimes give positive reactions with syphilitic serums. Nor is this unexpected. Since so many normal tissues serve as efficient Wassermann test antigens, it is not surprising that test antigens, prepared from heavily infected organs or even from the organisms themselves, should give pseudo-positives with syphilitic serums. This possibility should always be borne in mind in studying specific complement fixation in parasitic infections, especially when the test antigen is an alcoholic extract of organs, and should be carefully eliminated in devising various techniques.

2. AGGLUTINATION

The serums of many normal and immunized animals (natural and immune agglutinins) possess the *in vitro* property of causing uniform suspensions of bacteria, red blood-cells, and certain protozoa to form clumps which gradually fall to the bottom, leaving the diluent clear. Immune agglutinins often react in very high dilutions, and are highly specific, although they may react, generally to a less degree, with closely related organisms. The reaction has been used chiefly as a test for infection or as a means of identifying or studying the relationships of different organisms. The extensive investigations of Bull (1915, 1916, 1916 b; see also review by Bailey, 1928) indicate that the process *in vivo* may play a significant part in the natural and acquired immunity of animals to certain infections.

Some of the properties of agglutinins and their nature have been ascertained by numerous investigators, even though they have not been isolated apart from serum. They withstand heating to 60° C. for thirty minutes and resist drying. They do not dialyze through

animal membranes and are closely associated with the globulin fraction of serum, since they are precipitated from it by saturation with magnesium sulphate or half saturation with ammonium sulphate. Furthermore, if, before mixing, both they and the suspension of organisms are dialyzed free of salts, the reaction does not occur (Bordet, 1899). For a discussion of the mechanism of agglutination, the reader is referred to the recent review by Northrop (1928).

Other substances, such as acids, certain vegetable substances (ricin and abrin), etc., are also capable of agglutinating organisms, but these will not be considered further. Moreover, saline by itself is sometimes active in this respect. It is extremely important, therefore, to set up a control tube in the actual test containing simply the organism and whatever diluent is used, for, if agglutination occurs there, the whole test is invalidated.

Specific agglutination may be used (1) to ascertain whether an animal is infected with a certain organism, in which case the serum of the animal in varying dilutions is mixed with a known culture of bacteria, or (2) to ascertain whether the organism under examination is identical with one used in obtaining a particular agglutinating serum, in which case the organism is mixed with an agglutinating serum produced with a known organism. In either case, the actual test may be carried out by the macroscopic or microscopic method. In the tests, it is necessary to have a homogeneous, practically transparent suspension of microörganisms which is free of clumps. The protocol which is given in Table 5 and is performed

TABLE 5

PROTOCOL FOR AGGLUTINATION TEST

Tubes	*Antiserum*			*Suspension of organism*	*Diluent*
	Optional dilutions		*Amounts*		
			c.c.	c.c.	c.c.
1	1 : 10	1 : 5	0.5	0.5	0
2	1 : 20	1 : 10	0.5	0.5	0
3	1 : 40	1 : 20	0.5	0.5	0
4	1 : 80	1 : 40	0.5	0.5	0
5	1 : 100	1 : 80	0.5	0.5	0
6	1 : 120	1 : 160	0.5	0.5	0
7	1 : 140	1 : 320	0.5	0.5	0
8	1 : 160	1 : 640	0.5	0.5	0
9	1 : 180	1 : 1,280	0.5	0.5	0
10	Control		0	0.5	0.5

Mix and incubate 1 hr. at 37°C., refrigerate 12 hrs. and record the results (macroscopically or with a hand lens).

in 8 × 100 mm. tubes has been used extensively in experimental titrations. (Dilutions may of course be extended indefinitely, but should be high enough to rule out natural and group agglutinins, should they exist. They are usually made with saline, but sometimes with distilled water.)

The microscopic method for the agglutination reaction has been developed where only a small amount of blood serum or dry blood is obtainable. It is most widely used for the Gruber-Widal reaction for typhoid fever, and may be outlined as follows:

Cover slip	*Blood serum*		*Homogeneous moderate suspension of young bacterial culture*	*Saline*
	Dilution	Amount		
1	1:20	Platinum loopful	Platinum loopful	
2	1:40	Platinum loopful	Platinum loopful	
3			Platinum loopful	Platinum loopful (control)

Mix and invert each cover slip over a vaselined hanging-drop (concave) slide. Place in the dark. At the end of one hour examine with a microscope for clumping and motility, being sure that the control slide shows none. With dried blood, the procedure is essentially the same except that the blood which has been dried (preferably on a glass slide) can only be approximately diluted.

3. PRECIPITATION

This reaction is essentially the same as agglutination except that it is carried out with dissolved materials of bacteria, blood-cells, protozoa, etc., instead of whole cells, and is manifested by the formation of a precipitate instead of the clumping of cells. Therefore, what has been said of agglutination equally applies to precipitation. Moll (1906), Welsh and Chapman (1908), and subsequent workers have shown that the precipitate originates mainly from the immune serum.

Precipitin tests may be performed by observing the flocculation in a series of mixtures of test antigen and antiserum in which (1) the amounts of test antigen are held constant and the amounts of antiserum are varied as in the protocol for the agglutination test, or (2) the amounts of test antigen may be varied and the amounts of

antiserum held constant. The relation of the results of these two methods of titration to the actual precipitin titer of the antiserum is complicated by a number of factors, for a discussion of which the reader is referred to Cromwell (1925).

Where test antigen or serum is at a premium, clear-cut reactions can often be obtained with a simple "ring" test. This consists in carefully layering about 0.1 c.c. of the lighter fluid (generally the antigen) onto a like amount of the heavier fluid (generally the antiserum) in small 5 × 50 mm. tubes. A positive test shows a cloudy white precipitate (of various thicknesses according to the intensity of the reaction) at the interphase of test antigen and antiserum. Appropriate controls of each of the reagents with the particular diluent used should, of course, be set up and remain negative. The following is the typical protocol which I have used in various malarial and helminthological studies. (Further dilutions of the test antigen may be added if desired. In some cases, the tubes are incubated at 37° C. for one hour; in others, they are left at room temperature for varying lengths of time.)

Tube	*Antiserum undiluted*	*Antigen*		*Diluent*	
		Dilution	Amount		
1	0.15 c.c.	undiluted	0.15 c.c.	0 c.c.	
2	0.15	1 : 5	0.15	0	
3	0.15		0	0.15	Controls
4	0	undiluted	0.15	0.15	Controls

4. NEISSER-WECHSBERG PHENOMENON

In the preceding discussion the method of titrating each serological reagent has consisted in varying the one to be titrated while holding standard units of the others constant, with the result that the smallest amount or concentration of the reagent giving the desired effect represents the titer. Very frequently, however, in all such *in vitro* titrations, it is found that amounts larger than the effective one will inhibit the reaction. The inhibitory action of an excess of a given reagent in test-tube experiments was discovered by Neisser and Wechsberg (1901) who, during the estimation of the bactericidal property of immune serums, made the observation that in the presence of a constant amount of complement and antigen (*Vibrio metchnikovi*),

medium amounts of antiserum were completely bactericidal, whereas very large amounts of antiserum were no more bactericidal than small amounts.* Similar findings may be encountered in any serological titration and are exemplified in Table 6.

TABLE 6

THE ZONE PHENOMENON IN A PRECIPITIN TEST USING SHEEP SERUM AND SERUM FROM A RABBIT IMMUNIZED WITH SHEEP SERUM (FROM ZINSSER, 1923)

Test antigen (sheep serum)	*Antibody (anti-sheep rabbit serum)*	*Amount of precipitate*
c.c.	c.c.	
0.5 of 1 : 10	0.5	±
0.5 of 1 : 100	0.5	+++
0.5 of 1 : 500	0.5	+++
0.5 of 1 : 1000	0.5	++
0.5 of 1 : 5000	0.5	+
0.5 of 1 : 10000	0.5	—

This phenomenon is known under various names, one of the commonest of which is the *Neisser-Wechsberg phenomenon.* In reactions involving complement, it is often known as *complement deviation.*† This term arose from the fact that Neisser and Wechsberg explained their findings by assuming that where there was an excess of antibody (amboceptor), some united with the complement in the system and some united with the antigen; and in consequence, as both antigen and complement were separately bound, there could be no antigen-antibody-complement complex formed. There is, however, little or no evidence that the amboceptor can react directly with complement without previously combining with antigen, and most modern workers explain the phenomenon on the basis of physical or colloidal chemistry. (See Wells, 1925.) Other names which are used are *prezone phenomenon, zone phenomenon,* and *zones of inhibition.*

* A similar phenomenon was described by R. Pfeiffer (1895) and others in animal experiments before the work of Neisser and Wechsberg and was considered by them to have the same basis. These *in vivo* experiments will be considered in the discussion of the action of immune serums in the body (page 156).

† This term is often incorrectly used as synonymous with *complement fixation.*

5. THE ABDERHALDEN REACTION

Before leaving the general question of immunity reactions, mention should be made of the Abderhalden reaction, which is based on the general hypothesis that when foreign proteins are introduced parenterally, the body responds by forming specific protective ferments which destroy the proteins by proteolysis. That it is similar to some of the immunity phenomena is indicated by the fact that inactivation of the serum follows heating, and reactivation ensues on the addition of normal serum. It was introduced for the diagnosis of pregnancy, but theoretically could be used for infection or any condition in which foreign proteins are introduced parenterally.

In the test, the serum is incubated with the antigen under suitable conditions, with the result that very minute quantities of the products of protein cleavage may be set free, and may be recognized when dialyzed from the digesting mixture, or when an optical rotation of the digestive mixture in a polariscope occurs. (See Wells, 1925, and Bronfenbrenner, 1928.) The technique, so simple in theory, has to be carried out with meticulous care and regard for controls.

As far as I am aware, studies on this reaction are limited to two papers which deal with both protozoan and metazoan parasites. It seems best, therefore, to consider them here instead of under the specific infections. In interpreting this work, it must be remembered that if the central hypothesis is accepted, two types of reaction can be obtained: (1) there may be specific protective ferments for the parasites, and (2) if there is any destruction of tissue by the disease processes, with the consequent liberation of tissue disintegration products, there may be specific ferments for these tissues. Thus, if the reaction is based on sound reasoning, not only can the infection be detected by using a parasite substrate, but the extent of organ involvement can be ascertained by using a normal tissue substrate.

In the first paper, Gózony (1914) found that the serum of rabbits, guinea-pigs, rats, and man infected with various species of trypanosomes gave positive tests (group reactions were evinced) with liver and lung tissue from infected animals, whereas normal serum did not react, nor did infected serums with normal tissues. With *Sarcocystis* antiserum, positive reactions were obtained against the protozoa but not against muscle; with *Fasciola hepatica,* not against the worm (i.e., both normal and infected serums reacted), but against the muscle; and with *Trichinella,* against both the worms and the muscle. No cross-reactions were detected between the try-

panosomes and *F. hepatica* nor between the latter and *Sarcocystis*. In the second paper, Hindle and Gózony (1914) continued the work on piroplasms. In calves infected with *Theileria parva,* the serum gave positive reactions not only with isolated parasites, but with almost all organs. Since the reaction with organ tissues is supposed to follow the destruction of organ tissues with the consequent stimulation of ferments, these authors attempted to correlate the reaction with the progressive organ involvement due to the disease. Thus, in the early infection, positive reactions with infected serums were obtained with only the lymphatic and suprarenal glands, whereas in later stages they occurred with almost all organs. Similarly, in the early stages of infections with *Babesia canis,* positive tests were obtained with only liver tissue, but later in the disease with practically all organs.

II. *Amœbiasis*

Although there are a number of indirect signs presumptive of amœbic dysentery, diagnosis is not complete until *Endamœba histolytica* is found. This can usually be accomplished by a single stool examination in untreated acute amœbic dysentery, but is decidedly more troublesome in the carrier condition and in chronic and treated cases. In fact, in one extreme case, cited by Dobell (1917), the parasite was not found until the thirty-fifth consecutive examination. In routine survey work, one examination generally discloses only one third of the infections, while three examinations reveal one half to two thirds of the infections; from this, Dobell (1917) concluded that six negative examinations were necessary before pronouncing a case negative. In view of such attendant difficulties, an extensive study of various immunological reactions might well be repaid with practical usefulness.

Probably the first investigator to study the serology of amœbiasis was Izar (1914), who used as test antigen aqueous extracts of fecal material and liver abscess pus from a case of amœbic dysentery in the cat. He reported positive complement fixation with serums from five infected persons and three infected cats. Hage (1920), on the other hand, obtained negative results, and decided that the source of the test antigen contained too small a concentration of reactive material. Scalas (1921), in attempting to improve Izar's method, used as test antigen an aqueous extract of mucous flakes from positive stools, instead of the unselected feces, and obtained positive fixation with serums from three persons with amœbiasis (two of long standing and one mild case of eight months' duration) and negative

fixation with serums from a bacillary dysentery patient and a normal person.

By far the most valuable report on complement fixation is the recent study by Craig (1927 and 1928). He obtained his test antigen by extracting the sediment from forty-eight hour cultures of *E. histolytica,* grown on the Boeck-Drbohlav's medium with seven to ten volumes of absolute alcohol, incubating at 37° C. for ten days with several daily shakings and filtering. Of the 225 persons whose stools were examined by microscopical and cultural methods for amœbæ, serums from twenty-four infected and two uninfected gave positive complement fixation, whereas serums from two infected and 197 uninfected gave negative fixation. In general he concluded that complement fixation occurs not only in dysenteric patients, but in mild cases and "carriers," and that it does not occur in infections with *E. coli, E. nana, Iodamœba williamsi, Trichomonas hominis,* or *Chilomastix mesnili,* nor in various other diseases including syphilis, unless *E. histolytica* is also present. He seems inclined, however, to withhold judgment on the practical significance of the reaction because of the technical difficulties in carrying out the test

In studying precipitins in cats infected with *E. histolytica,* Wagener (1924) prepared her test antigen by placing scrapings of ulcerated intestines from infected cats in Coca's extracting fluid (NaCl 0.5 per cent, $NaHCO_3$ 0.05 per cent, phenol 0.4 per cent) for several days, and filtering through hard filter-paper. "Ring" tests were conducted. In each case, control tests were set up with test antigens prepared from the bacteria found associated with the amœbæ in the scrapings, and these were consistently negative. Twenty-five cats,

TABLE 7

PRECIPITIN RESULTS WITH THE SERUMS OF NORMAL CATS AND THOSE IN VARIOUS STAGES OF INFECTION WITH ENDAMŒBA HISTOLYTICA (DATA FROM WAGENER, 1924)

Cats	*Stage of infection*	*Precipitin results with various dilutions of serum*				
		undil.	*1:1*	*1:2*	*1:4*	*1:8*
1	Dying with no lesions	—	—	—	—	—
6	Discharging amœbæ less than 6 days	—	—	—	—	—
3	Discharging amœbæ 6-8 days	+	—	—	—	—
7	Discharging amœbæ 8 or more days	+	+	+	+	—
2	Chronic infection of 40 days	+	—	—	—	—
1	Recovered case	—	—	—	—	—
5	Normal	—	—	—	—	—

twenty of which had or had had the infection, and five of which were normal, gave the results shown in Table 7. These results, in conjunction with certain others in her paper, indicate that the reaction is specific and worthy of further study.

III. *Leishmaniosis*

Most of the work on the serology of the *Leishmania* infections has been directed toward either the development of a serological diagnostic test for the visceral infection, kala-azar (*L. donovani*), or the classification or differentiation of the different species.

Although there is every indication that agglutinin, precipitin and complement-fixing antibodies can be demonstrated in animals artificially immunized with cultures of *L. donovani,* and to a certain extent in infected humans, as yet there is very little promise that they will be of any practical importance in diagnosis. With the exception of a few authors, most investigators have either gotten negative results or such low titers and such low percentages of positives that it is out of the question to use the tests in practical diagnosis, although further work on technique might prove worth while. In contrast to these results with specific antibodies, various investigations indicate that certain miscellaneous serum reactions will actually yield methods of tremendous practical importance, as have the non-specific reactions for the diagnosis of syphilis.

In reviewing this work, I shall consider the work on specific antibodies first, and then the miscellaneous serum reactions. Furthermore, as comparatively little work has been done on the cutaneous leishmanioses, I shall include it with that on kala-azar.

1. COMPLEMENT FIXATION

As in the other protozoan infections, the work on complement fixation has been concerned with both non-specific and specific test antigens. Tests in the first category, which amount to a study of possible pseudo-positives in the Wassermann reaction, have been practically negative where syphilis could be definitely excluded. (See, for example, the results of McKinstry (1922) on oriental sore, Leão (1922) on South American leishmaniosis, and Iyengar (1923) on kala-azar.) Those with specific antigens were mostly negative at first, but a few recent investigations have given more promising results.

The early work on specific complement fixation in kala-azar by Jemma and di Cristina (1911) and by Longo (1911) were negative.

As test antigens, the first used aqueous and the second alcoholic extracts of splenic pulp from children dead of kala-azar.

Makkas and Papassotiriou (1911) used a Wassermann test antigen, with which they obtained negative results for kala-azar, and an aqueous extract of an infected spleen from a ten-months-old child that had died of kala-azar (Ponos), with which they obtained the following results: serums from five cases of kala-azar, three of syphilis, and two of malaria were positive, but those from two healthy persons were negative. The authors suggested that by combining the two tests a diagnosis could be made, but they pointed out the advisability of more extensive work.

Closely following this work, di Cristina (1911 b) obtained negative complement fixation with the serums of three rabbits before immunization with cultures, and positive fixation after immunization. His test antigen consisted of a heavily infected spleen triturated, dried *in vacuo,* pulverized, and extracted with saline. But in a later paper (1912), he was unable to check the results of the previous investigators, using his known reactive test antigen (positive with immunized rabbits) on seven serums from infected humans. Similar negative results were reported by Longo (1912) in three children in the second stages of kala-azar and in three non-infected persons. In line with the positive results after immunization, di Cristina and Caronia (1912) reported work on seven infected persons who were negative for complement fixation, but became positive following injections of cultures killed by heating at 55° C. In a second paper (1912 b), positive complement fixation was obtained in two cases of spontaneous recovery where it had been negative twice before (i.e., when parasites had been found, and after treatment). The authors concluded that recovery was associated with the formation of antibodies. In the same year, one of the authors (Caronia, 1912) reported the case of an uninfected child whose serum showed no agglutinins nor complement-fixing antibodies before immunization, nor after five daily subcutaneous injections of 1 c.c. of dead cultures, but was positive after the treatment had been continued six more days. Further work (Caronia, 1913), showed that eight cases, having had kala-azar from one to five months, gave negative complement fixation tests, but became positive after a period of immunization. In summarizing the work done at the University of Palermo, di Cristina and Caronia (1913) stated that in complement fixation tests on eighty-eight cases of infantile kala-azar, partial or definitely positive tests were obtained in only thirteen cases. During the course of this work, they at-

tempted to use as test antigen an alcoholic extract of fleas from an infected dog, but did not obtain very promising results.

Pavoni (1914) found strong fixation of complement in two cases of oriental sore, but almost always obtained negative results in kala-azar exhibiting a normal course. Fixation did occur, however, in recovered cases of kala-azar and sometimes in chronic cases, but in the latter it tended to disappear if the disease suddenly became worse. In all, the author investigated thirty-four cases of kala-azar. Control tests on forty-five children and twenty-five adults were all negative. The test antigen consisted of an aqueous extract of an infected spleen or cultures of *L. infantum*. Cornwall and La Frenais (1916) failed to obtain complement fixation in one case of kala-azar.

Brahmachari (1917) used as test antigen the supernatant obtained after heating one part of freshly ground kala-azar spleen for an hour at 60° C. in three parts of a mixture of half alcohol and half 0.85 per cent NaCl. In six out of eight cases, fixation was obtained. Negative results were reported in two cases by Olsen (1918) and in four by R. Knowles (1920). Kasuga and Tamura (1925) obtained a certain degree of fixation in immunized animals.

Much more promising results were reported by Hindle, Hou, and Patton (1926), who prepared their test antigen by triturating the spleen of a very heavily infected hamster in 5 c.c. of saline, straining through muslin and adding 1 per cent glycerin and 0.5 per cent phenol. In carrying out the test, 0.8 c.c. of this suspension was used with 0.2 c.c. of inactivated patient's serum. Of twenty-four cases of kala-azar which had been diagnosed by liver puncture, they obtained well-marked complement fixation in nineteen and partial in five, while in fifty-four patients suffering from other diseases, six showed fixation (five luetic, one anticomplementary), four partial and forty-four none. This work again suggests that, with a suitable test antigen and careful technique, the reaction might be perfected for diagnostic measures.

Similarly, Auricchio (1927), using an antigen prepared from flagellates of ten-day to twelve-day cultures (flagellates from one tube suspended in 2 c.c. of physiological saline), studied twenty-four cases of the Mediterranean disease. Twenty-four tests on patients suffering with the disease for from one to seven months gave 9+++, 9++, 3+ and 3—, whereas eight tests on patients improved or cured gave 1+++, 1++, 3+ and 3—.

Comparatively little has been done on complement fixation in the two dermal leishmanioses (*L. tropica* and *L. brasiliensis*), but the

work that has been done suggests that complement fixation is very much easier to obtain than in kala-azar. Pavoni (1914 and 1914 b), using as test antigen an aqueous extract of kala-azar spleen, obtained positive results in one case of dermal leishmaniosis and in two cases of oriental sore, although practically negative results were obtained with the homologous kala-azar serums.

Working with the South American dermal leishmaniosis, Moses (1919) used an aqueous test antigen prepared from cultural flagellates and obtained positive results in 80 per cent of forty-one cases (eighteen untreated and twenty-three treated or cured), a group reaction in an animal infected with trypanosomes, and negative results in various bacterial diseases.

Besides the work just reviewed, which deals with the detection of antibodies in infected persons, several investigators have found it comparatively easy to obtain specific complement fixation in animals immunized with leishmanial products. As this work was largely done to study the systematic relationships and affinities of the organisms, it is reviewed in Chapter IX.

2. AGGLUTINATION

The remainder of the work on specific antibodies in the leishmanioses has been chiefly on agglutinins, although some investigators have studied precipitins and leishmanicidal antibodies. The results are just about as contradictory as are those on complement fixation in infected persons, but there is no question that such antibodies occur in high titers in animals immunized with leishmanial products.

The early work of Nicolle and Manceaux (1909 and 1910) failed to show either leishmanicidal or agglutinating antibodies in two experiments with the serum of a dog heavily infected with *L. donovani* and tested against fifteen-day cultures of the parasite. These investigators mixed equal parts of the dog serum and the cultural flagellates, kept the mixture at room temperature, and examined it at intervals, but found no indication of agglutination or destruction of the flagellates. Similar failures were noted by Jemma and di Cristina (1911), di Cristina (1911), and Longo (1912), in testing the serums of infected infants for agglutinins.

The first clear-cut demonstration of specific agglutinins was found in artificially immunized animals by di Cristina (1911 b). He obtained specific agglutinins in the serums of rabbits immunized by intravenous injections of cultures of *L. donovani*. The titer was,

however, very low, being only 1 : 30. Caronia (1912) obtained similar results in a child immunized by subcutaneous injections of killed cultures of *L. donovani*. Following this work up to the present time a series of investigators have produced specific agglutinins in animals and man by immunization. These will be considered in Chapter IX.

Caronia (1913) studied the agglutinins and precipitins in children naturally infected with *L. donovani*. Serums from four out of five of these gave titers of 1 : 20 to 1 : 30 for agglutinins and 1 : 10 to 1 : 30 for precipitins, whereas eight other infected children gave negative results at first, but after six of them were immunized with killed cultures of *L. donovani* and two with nucleo-protein extracts of cultures, their serums gave titers of 1 : 25 to 1 : 100 for agglutinins and 1 : 25 to 1 : 50 for precipitins. Hence, he concluded that although specific agglutinins and precipitins occurred in the blood of persons affected with kala-azar, they were too weak to be used in diagnosis. Di Cristina and Caronia (1913) showed that serums from two healthy as well as from two infected children, after immunization, gave positive reactions. Scordo (1914) likewise found that serums of kala-azar patients had some agglutinative action on cultural forms of *Leishmania* and were more potent against cultures of human than of canine origin. Archibald (1914), Cornwall and La Frenais (1916), Olsen (1918) and Auricchio (1927), on the other hand, were unable to obtain agglutinins or precipitins in kala-azar patients.

In all of this work on the serum reactions of persons or animals infected with *Leishmania*, when the titers are very low, great care must be used to rule out the action of normal serum. Thus, a leishmanicidal property of normal human serum was noted by Archibald (1914). When the serum was added to washed cultural flagellates, the organisms gradually became immobile and finally were destroyed. According to Cornwall (1916), it was found not only in human but in goat, sheep and guinea-pig serum, was destroyed by heating to 55-56° C. and was not possessed, or only slightly so, by rabbit, dog, hen and cat serum. It could only be demonstrated in normal or infected persons, however, by Olsen (1918), and when destroyed by heat could not be reactivated with complement. R. Knowles (1920) called the property an "immobilin" because of the initial immobilization of the flagellates. He found, contrary to Olsen, that complement could reactivate heated serum and also discovered that the property was present in fresh syphilitic serum. Recently, Hindle, Hou and Patton (1926), in a series of experiments on this property with hanging drops, obtained quite variable results. Out of forty normal human serums, sixteen failed to immobilize the parasites;

and out of thirty-nine kala-azar serums, fourteen failed to do so. In serums from hamsters, dogs, sheep, rabbits and guinea-pigs, there was evidence of some immobilization and destruction of flagellates, and by increasing the amount of serum, the majority could be affected; but, on the whole, the results were also inconstant. Inactivated serum was ineffective, as was cerebrospinal fluid. Some of the foregoing investigators had concluded that the flagellate stage could not be the form which invaded the body; but, as Hindle, Hou and Patton pointed out, the few forms which are able to withstand the serum's effect might be responsible for a subsequent infection.

3. MISCELLANEOUS TESTS

1. *Brahmachari's serum-globulin test.* In a study of various blood reactions in kala-azar, Brahmachari (1917) noted that the addition of an excess of distilled water to serum from kala-azar patients often resulted in the formation of a copious precipitate of globulin-like material. A solution of this precipitate in physiological saline was anticomplementary in a hemolytic system. Continuing this work he (1917 b) found that whereas an excess of distilled water produced the precipitate, not only in kala-azar, but in other diseases, if only two or three volumes of distilled water were added, the precipitate appeared almost exclusively in kala-azar. Ring tests could also be made by first diluting the serum 10 to 20 times with saline in a test-tube and then layering distilled water on top of the diluted serum. Tests were carried out on twenty kala-azar patients with positive results and on twelve controls, having various diseases other than kala-azar, with negative results. The author also found a few cases which clinically appeared to be kala-azar but did not show *L. donovani* in spleen smears, and yet yielded positive tests. When the precipitate was dissolved in serum, the anticomplementary action, previously noted, did not occur.

The same phenomenon is concerned in the "hemolytic test" devised by Ray (1921) for kala-azar. In using a Gower hemoglobinometer Ray noted that a clear solution could not be obtained with blood from kala-azar patients. The author ascribed this to the incomplete hemolysis of the red cells, but stated that it depended on the serum and not on the red cells, because if the latter were washed and resuspended in normal human serum they hemolyzed in the usual manner. In the actual test suggested for kala-azar two drops of blood were placed in a test-tube and twenty drops of distilled water added. In positive reactions the liquid became turbid on

standing and a flocculent precipitate appeared. The reaction was present in serums from fifty-five cases of kala-azar and was negative in serums from normal humans and from those suffering from other diseases.

Sia (1921) modified Ray's test by mixing 0.02 c.c. of blood with 0.6 c.c. of distilled water in test-tubes of 7 to 8 mm. bore, and reading for turbidity or a flocculent precipitate after five to ten minutes. In sixteen kala-azar cases, eighty-six patients with other diseases and ten normals, he obtained a perfect correlation, but found that upon improvement in the kala-azar cases, the turbidity decreased. Furthermore, since anemias of various kinds seemed to give a certain percentage of false positives, it is of interest that serums from ten patients with anemia, with various hemoglobin readings of 30 to 60 per cent, gave negative tests. In a second paper, Sia and Wu (1921) found that serum from cases of kala-azar did not protect red cells from hemolysis by saponin. Moreover, they found the globulin constituent of serum was markedly increased in kala-azar and was responsible for the precipitate. In consequence, they proposed to call the test the globulin precipitation test for kala-azar, which definitely identified it with the test previously described by Brahmachari.

Working in Italy, Milio (1922) found Brahmachari's test positive in fourteen cases of kala-azar and negative in a like number of controls. In performing the test this author placed 1 c.c. of serum in a Wassermann tube and overlaid it with 2 c.c. of distilled water. A ring of turbidity at the interphase of the two liquids indicated a positive reading. Nasso (1923), however, also working on the Mediterranean disease, came to the conclusion that Brahmachari's test was of no value in that it was difficult to interpret and was positive in anemias not due to kala-azar.

With such a striking precipitation on the dilution of kala-azar serum with distilled water, it would be strange if nobody had previously observed a similar phenomenon when diluting kala-azar blood with various liquids. I am not aware of the frequency of the observation, but Wenyon (1922) pointed out that probably it explains an early observation by Hill (1913) that when kala-azar blood was diluted with his particular diluent (Wright's blood stain, 1 part, and 0.1 per cent NaCl in distilled water, 3 parts) used in making leucocyte counts, the red cells, instead of disappearing immediately as in other diseases, ran together in clumps which could be broken up only by long and vigorous shaking. Hill described this as a new sign of kala-azar.

With such promising preliminary results, attempts have been made to put the test on a quantitative basis. Thus, Brahmachari and Sen (1923) have devised the following method for estimating the amount of water-precipitable globulin in the serum: The serum is mixed uniformly with six times its volume of distilled water, and poured into a graduated cylinder with a diameter of one inch. Some black spots are fixed at the bottom of the cylinder and the serum mixture poured in until the spots are just visible. The height in inches varies inversely with the amount of precipitate; a reading of 1.25 inch or less is regarded as positive. Sia (1924) again advocated the quantities he had used in 1921, and classed the reactions as strongly, moderately or weakly positive according to whether a sediment was formed in fifteen, thirty, or sixty minutes, respectively; very weakly positive when no sediment, but a fine precipitate, appeared after one hour; and doubtfully positive when only haziness occurred after one hour.

2. *Aldehyde (formol gel) test.* The original formol gel test was proposed by Gaté and Papacostas (1920) for syphilis. When positive it consisted of a jellification of syphilitic serum upon the addition of commercial formalin (1 c.c. of clear serum to 2 drops of formalin) after the mixture had stood at room temperature twenty-four to thirty hours. Several authors, including the discoverers, have since shown that the test is not of much value in syphilis. Thus, Gaté and Papacostas (1922) found that in syphilis and erysipelas there were roughly 50 and 100 per cent positives respectively, while in typhoid fever, pneumonia and normals there were generally about 100 per cent negatives. Bessemans (1922 c and d) studied the effect of various conditions in making different serums undergo jellification upon the addition of formalin. The power to form a jelly was increased by concentration, by evaporation and by the addition of certain salts, such as ammonium and magnesium sulphate and sodium chloride. Combiesco (1922) similarly found the test positive in erysipelas and scarlatina and believed that the reaction depended upon an instability of the colloidal substances of the serum. He added the curious fact that guinea-pig serum was negative when normal, but gave a positive test during the anaphylactic state and was particularly marked during anaphylactic shock.

The fact that the test was originally devised for syphilis and found positive in a number of conditions has led some investigators to deny its value in kala-azar. They overlook the fact, however, that the so-called formol gel test for kala-azar does not depend solely on the formation of a jelly as in Gaté and Papacostas' test, but on

the formation of a copious precipitate causing the serum to become quite opaque like coagulated white of egg. Thus, the kala-azar test uses an entirely different criterion. This will be brought out in the discussion that follows.

In 1921, Spackman noted that the serum of kala-azar patients jellied in a few seconds instead of the long period set by Gaté and Papacostas, and moreover, that it became opaque. Napier (1921) used the test on fifty proved cases of kala-azar, forty-nine of which exhibited jellification and opacity in thirty minutes. Fox and Mackie (1921) devised a simple modification in which the patient's blood was collected in a Wright's capsule and allowed to clot. Then a drop of serum was placed on a slide and inverted over a watch-glass containing several drops of formalin. Fumes of the formalin would, within a few minutes, cause the serum from a kala-azar case to become opaque and form a stiff jelly which adhered to the slide. They obtained positive results with serums from twenty proved kala-azar patients, two syphilitic and one malarial patient (suspected also of having kala-azar) and negative results with serums from seventeen syphilitic and three malarial patients and six normals.

A very much more detailed study of the test was made by Napier (1922) who considered a positive as consisting of the formation of a solid gel with a dead white opacity resembling coagulated white of egg in from three to twenty minutes (in rare cases, two to twenty-four hours). As the opacity is really the characteristic criterion for the test, Napier suggested that it should be termed the aldehyde test and not a formol gel reaction, thereby avoiding confusion of this test with the formol gel test in syphilis which has fallen into disrepute. The actual chemistry of the test is probably unknown, but Napier found that one drop of a specially prepared pure solution of 10 per cent formaldehyde gave better results than the stronger commercial formalin when used in the same proportion, and he considered it essentially an aldehyde reaction because the reaction is given with acetaldehyde, although much more slowly and less completely. In carrying out the test, 5 c.c. of blood is drawn by venipuncture, 1 c.c. of the separated serum placed in a small test-tube (about 12 mm. in diameter) and 1 drop of commercial formalin (30 per cent formaldehyde) added to it; the mixture is then shaken and kept at room temperature. Within a minute, in kala-azar serums, the serum will have set so that the tube can be inverted. The serum will also begin to show a whitish opalescence which within three to twenty minutes becomes absolutely solid and opaque, as described above. Where there is hemoglobin in the serum the pink tinge changes

to a chocolate brown in twenty-four hours. With serums from 150 cases suggesting kala-azar the test gave a 98 per cent correspondence with the findings by spleen puncture. Of particular interest is the comparison of the Wassermann and aldehyde tests in eighty-six of these cases. Of sixty which gave a positive aldehyde test the Wassermann tests were as follows: twelve positive, seven partially positive and forty-one negative. Of twenty-six which were negative for the aldehyde test the Wassermann tests gave four positive, five partially positive and seventeen negative. Of various diseases other than kala-azar, only the serums from patients with tuberculosis or leprosy exhibited any clouding. A study of twenty-nine cases undergoing treatment indicated that the reaction tended to disappear as the treatment progressed and this suggested that the test might be useful in evaluating the efficacy of the treatment. Among various modifications designed to use small quantities of blood (none of which are as good, however) the following was found to be the best. Four drops of blood from the finger are dropped into 0.5 c.c. of a solution containing sodium chloride, 0.85 gm.; sodium citrate, 0.5 gm.; water, 100 c.c.; and one drop of formalin. When left over night the blood cells settle out and the supernatant is milky in kala-azar cases and clear in others.

Immediately following Napier's article one by Mills (1922) recorded an absolute correspondence between the test and infection in eleven cases. Ganguly (1922) noted that in some proved cases of kala-azar the jellification occurred in ten minutes, but in only six was it complete in five minutes, and that it often occurred before opacity. One case of syphilis was noted in which complete solidification and opacity were present in ten minutes.

In a further study, Napier (1923) made an extensive study of 353 persons, of whom 114 were hospital patients and 239 were outpatients. He classified the reactions as follows:

Positive	+++, serum solid and completely opaque in 20 minutes
	++, serum solid and completely opaque in 2 hours
	+, serum solid and completely opaque in 24 hours
Doubtful	(+), serum markedly opalescent and solid, but not completely opaque
	(±), serum solid and slightly opalescent
Negative	(−), serum solid, but clear
	−, serum fluid and clear for 24 hours

TABLE 8

CORRELATION OF ALDEHYDE TEST FOR KALA-AZAR AND RESULTS OF SPLEEN PUNCTURE (DATA FROM NAPIER, 1923)

Spleen examination	*Aldehyde test* +	?	—	*Totals*
+	191	32	8	231
—	10	16	96	122*
Totals	201	48	104	353

* Of these 122 patients negative by spleen puncture, 14 were hospital patients and had the following examinations: 1 had a single spleen puncture, 8 had two, 4 had three, and 1 had four.

Combining all of Napier's tables, there is a remarkably high correlation between the results of spleen puncture and the aldehyde test (Table 8), especially when it is remembered that many of the so-called negative cases had only a single spleen puncture, so that some of them may have been positive. One very interesting point is brought out by Napier in regard to the 239 out-patients of whom 119 gave a positive aldehyde test. In these 119 patients one spleen puncture revealed parasites in 110, but previous work has indicated that one spleen puncture reveals only 90.8 per cent of the infected cases. Therefore, the results with the spleen puncture are just what would have been expected had all of the 119 individuals been infected as indicated by the aldehyde test.

With such beautiful results in the hands of the perfecter of the test it is interesting to consider some of the other workers' experiences. Sarkar and De (1923) believed that the action was much delayed in some cases, but as Wenyon pointed out, this conclusion is invalidated by the fact that the cases were not definitely diagnosed by finding the parasites and one third of the cases apparently underwent spontaneous recovery. In 1924, many papers reported satisfactory results. De Capite (1924) tried the test on fifty-seven persons comprising eighteen cases of kala-azar, two cases of recovered kala-azar, seven cases of splenic anemia and thirty cases of other diseases. He considered the test of enough accuracy to warrant a probable diagnosis. Six of the eighteen serums of kala-azar, however, took approximately six hours to become positive. There seemed to be, then, more delayed reactions than Napier described. In a study of seventy-five cases of kala-azar Ganguli (1924) concluded that the aldehyde test was more reliable than the globulin test and

was generally positive in three minutes and rarely took longer than twenty minutes. He noted a case of mild splenomedullary leukæmia which gave the reaction in three minutes. Elwes, Menon and Ramakrishnan (1924) reported that in eighty-one demonstrated cases of kala-azar 23.4 per cent of the tests were entirely negative and 12.3 per cent were doubtful. Thus, the reaction was practically of no value in 35.7 per cent of the cases. They also noted a number of cases giving positive reactions which clinically appeared to be kala-azar although no parasites were discovered by liver puncture. These they believed were not kala-azar and served to complicate the use of the aldehyde test for diagnosing the infection. In an appended note, however, Napier expressed the belief that the last assumption was not proved and noted that the test has its limitations, but is, on the whole, reliable. Working in China, Struthers and Ch'un (1924) applied both this and the globulin test to 141 cases, 140 of which were demonstrated to harbor *L. donovani* and the other of which showed the classical characteristics of the disease. With the formol gel test 98 per cent gave clear positives and the remaining 2 per cent were early cases (similar to Napier's findings), whereas with the globulin test 93 per cent were positive and 7 per cent negative. In sixty-seven other diseases the tests were uniformly negative. In a second paper, Struthers (1924) stated that neither test seemed reliable in indicating cure of the disease in consideration of the following results on thirty-seven apparently cured cases:

	+	?	—
Aldehyde test	17		20
Globulin precipitation	4	16	17

Ganguli (1925) believed that the test depended on an increase of the euglobulin content of the blood and that a suspected case of kala-azar giving a negative reaction after five months' illness could safely be assumed not to be kala-azar.

In evaluating the different methods for the diagnosis of kala-azar, Shortt (1924) considered culture from the liver and spleen the best, direct examination of smears of such puncture material next best, and the aldehyde test and examination of the peripheral blood third. R. Knowles (1928) stated, "The value of the aldehyde test, if Napier's criteria, that only the simultaneous presence of both gel and white opacity are to be taken as a positive reading, is very great in field and village work . . . On the other hand in the early months of the disease the test is of no practical value prior

to the fifth month of illness and it cannot in any way replace the final proof that the disease is kala-azar by the finding of the parasite either in films or cultures."

3. *Antimony test.* Chopra in collaboration with Gupta and David (1927) suggested still another flocculation test for kala-azar based on the fact that antimony derivatives, especially urea compounds, when brought in contact with kala-azar serum, seemed to produce an immediate thick, copious precipitate, whereas when brought in contact with other serums, only a slight precipitate or none at all formed. This test seemed to possess certain advantages over the aldehyde test. Smaller amounts of serum were required, a stock solution of urea-stibamine was efficient for ten days, and immediate positive results were obtained, even in early cases. Napier (1927) found that the efficacy of a number of pentavalent antimony compounds for this test varied directly with their therapeutic efficacy—von Heyden 693 giving the most intense reaction. He suggested carrying out the test by adding two drops of serum, which had been stored twenty-four hours, to 2 c.c. of a 0.25 per cent solution of urea-stibamine or two drops of fresh serum to 2 c.c. of a 1 per cent solution, agitating and allowing to stand for ten minutes. In positive tests at the end of this time a heavy flocculent precipitate settled out, leaving a clear supernatant. Chopra, Gupta and Basu (1927), unlike Napier, considered it unnecessary to keep the serum twenty-four hours and advocated stronger (1 to 4 per cent) solutions. They used two methods. The first consisted in placing one volume of serum in a small Dreyer's tube and overlaying with a like quantity of a 4 per cent solution of the drug (preferably urea-stibamine). A second, simplified modification utilized whole blood (two drops mixed with 0.25 c.c. of 2 per cent potassium oxalate), instead of serum. Some of this mixture was then placed in a second tube and a 4 per cent solution of the antimony compound allowed to flow along the side of the tube from a capillary pipette so that it underlaid the blood mixture. Although in strongly positive cases a precipitate formed at the junction immediately, in very early cases of kala-azar it sometimes took from ten to fifteen minutes—rarely one to two hours. Using the first method the authors tested serums from seventy kala-azar patients and found all strongly positive. Tests on fifty-nine controls yielded the following results: Serums from thirty-five lepers gave thirty-three negatives and two doubtful positives; from fourteen malaria cases gave thirteen negatives and one moderately positive; and from ten other cases gave nine negatives and one positive. Furthermore, it is interesting that of the seventy kala-azar cases

only sixty gave strongly positive aldehyde reactions. Using the second simplified test, fifty-four cases of kala-azar were positive and eighty-four controls with other diseases were negative. In a later paper, Chopra, Gupta and Basu (1927 b) pointed out that mistakes may arise if particular attention is not paid to the character of the precipitate which forms at the interphase of the serum and urea-stibamine solution. In kala-azar, this is flocculent, not easily broken up by shaking and persists for twenty-four hours; in some other diseases, it is non-flocculent. Also, some batches of drug seem to give a precipitate with normals, but preliminary tests with each newly made solution will rule these out. They advocated the dilution of the serum (eight to ten times) with distilled water in doubtful cases. In this paper, they reaffirmed the superiority of the antimony over the aldehyde test as the following table shows:

	Antimony test			*Aldehyde test*		
Kala-azar cases. (Duration)	+	?	—	+	?	—
10-12 days	0	3	0	0	0	3
15-21 days	12	0	0	0	0	12
1-3 months	30	0	0	0	4	26
Various lengths	235	21	0	184	40	32
Other diseases	18	26	181	10	39	176

Furthermore, although the numbers are small, the test seemed to remain positive through ten or eleven drug injections, then gradually became negative through thirteen to fifteen injections. This is interesting in view of the fact that Napier considers ten to fifteen injections sufficient to cure a patient. Sen (1927) applied the test to forty-two serums with the following results: twenty from kala-azar patients were positive, twenty-two non-infected cases gave eighteen negatives and four positives. Sanyal (1927) proposed a modification of the test which could be carried out during the process of injecting the antimonial for treatment. Just before the injection of the urea-stibamine or amino-stiburea, which is dissolved in distilled water, a drop of blood is aspirated into the syringe. Then the contents of the syringe are all injected except 0.1 to 0.3 c.c. After withdrawing the needle, this remainder is diluted by aspirating about 2-3 c.c. of distilled water into the syringe. In kala-azar the mixture is decidedly opaque and this opacity diminishes as successful treatment progresses. Working in Italy, Fabris (1928) obtained results

in agreement with the workers in India on twenty-five persons with kala-azar, sixty-five normals and 245 persons with various other diseases. In a critical examination of the aldehyde and antimony tests, Napier (1928) concluded that the latter is the more delicate, but even so is not absolutely accurate, so that he advised certain clinical signs to be used in conjunction with it.

4. *The miscellaneous tests used in kala-azar in relation to other parasitic diseases.* The increasing usefulness of the serum globulin, aldehyde (formol gel) and antimony tests in kala-azar makes the studies of other diseases,* as complicating factors, of practical importance. Such diseases may not necessarily be confusing when they are of restricted distribution and only accidentally occur in kala-azar areas. The criterion in all the tests for kala-azar, it will be remembered, is preëminently the clouding of positive serums due to globulin precipitation, with concomitant jellying in the formaldehyde test.

The results of E. C. Faust and Meleney (1924) indicate that the serum globulin is greatly increased in infections with *Schistosoma japonicum.* They used two of the tests, Sia's (1921) quantitative method for the globulin precipitation reaction and Napier's (1922 and 1923) method for the aldehyde test. Eighteen globulin precipitation tests on fifteen positive schistosome cases yielded 1 ++++, 2 +++, 9 ++, 3+ and 3 — reactions; twelve aldehyde tests on similarly positive serums, eleven of which occurred in the previous series, gave 1 +++, 5 ++, 5 + and 1 negative. Accordingly, when the two diseases are coincident, the test must evidently be used with care. In regard to the use of the tests for schistosomiasis where kala-azar can be excluded, the authors felt that it would not supersede fecal diagnosis but that it might prove instrumental in estimating the efficacy of drug treatment. (See also Meleney and Wu, 1924.)

Lal (1923) carried out some aldehyde tests in Lahore where kala-azar does not occur, with the serums from patients who were infected with malaria, but showed no signs of past or present cutaneous leishmaniosis. Although some of the tests showed opacity, none were definitely positive by Napier's standards.

Both opalescence and jellification were used by Spackman (1923) and R. H. Knowles (1924 and 1925) in testing animal trypanosomiasis. The former tested the serums from eight camels with the following results: six infected with *T. evansi* gave five positive and one moderately positive reaction, and the two uninfected gave negative reactions. In view of the correlation between treatment and re-

* Syphilis has already been considered (p. 40).

activity of the host in kala-azar, it seems worth pointing out that three of the infected camels had been treated and that the one which had received the largest amount of drug gave the moderate reaction. Knowles worked on camels infected with *T. soudanense* (*T. evansi*). Seven infected animals gave seven positives; twelve normal ones, one doubtful and eleven negative; two normal bulls, negative, and two normal horses, doubtful reactions (1924). Furthermore, he substantiated Spackman's observation on treatment. Two camels not treated and four treated from fourteen to forty-eight days all gave positive reactions, whereas five treated 7.5 to 9 months gave one positive, one partial and three negative reactions. A normal camel also was negative (1925). As far as I can ascertain, these tests have not been used in human trypanosomiasis.

There have been, however, a few papers on both animal and human trypanosomiasis using the Gaté-Papacostas test, in which the criterion is gel formation alone. Working with animal trypanosomiases, the test seemed unreliable, according to Hornby (1923), Plantureux (1923 and especially 1924) and van Saceghem (1925). In human sleeping sickness, greater success was attained. Morrison (1924) reported that ten infected, treated cases gave 9 + and 1 ?, while ten controls gave 3 +, 1 ? and 6 — reactions. W. B. Johnson (1925) tested 168 serums, but decided that the reaction was of little value in the trypanosome infections. Dye (1926) was more optimistic. His tests, in general, were positive in untreated cases infected with *T. rhodesiense,* and gradually became negative with treatment. Ledentu and Vaucel (1927) also reported promising results on seventy-four cases infected with trypanosomes, and in ninety-six others, either suspected of infection or normal. On the other hand, the test was pronounced valueless by Polidori (1922) in five cases of malaria and by Dye (1926) in malaria, filariasis and amœbiasis.

5. *Nature of the miscellaneous tests for kala-azar.* The fact that the chief characteristic of all the miscellaneous tests for kala-azar is the formation of a copious precipitate suggests that they all have a common basis. This, many workers on the subject believe to be, or to be closely connected with, the euglobulin content of the serum. A few selected studies will suffice to bring this out. The name "serum globulin test" indicates that Brahmachari believed the substance involved to be globulin; he later (1917 b) stated that the precipitate was globulin-like in nature because it was soluble in physiological saline, dilute acids, dilute sodium bicarbonate and sodium hydrate, but insoluble in distilled water; because it was precipitated from solution in physiological saline by saturation with sodium chloride

and magnesium sulphate or by half saturation with ammonium sulphate, while chemical analysis showed it to contain carbon, nitrogen, hydrogen and oxygen, although no sulphur, phosphorus or halogens were detectable. Napier (1922) in his original description of the aldehyde test, believed the euglobulin to be responsible, and furthermore suggested that the pH of the serum was a factor since it is much increased in kala-azar. In a later paper, Brahmachari and Sen (1923) concluded that the same material is responsible for the two tests. When the precipitate from a kala-azar serum, obtained by adding distilled water, was redissolved in a serum negative for the aldehyde test, the addition of formalin caused a positive aldehyde test. In addition, precipitation followed the addition of formaldehyde to a saline solution of the precipitate from a kala-azar serum, especially if the solution was made slightly alkaline with $NaHCO_3$. Ray (1924) found that although the serum euglobulin constitutes 7 to 8 per cent of the total globulin in normal serum, it rises to 40 to 50 per cent in kala-azar serum, and since it is insoluble in water he considers it responsible for all the miscellaneous blood tests (See also Ray, 1927). Similar conclusions were reached by Ganguli (1925) who found that the average nitrogen content of the euglobulin fraction in normals, malarial patients and early and advanced cases of kala-azar (four of each) were 0.031, 0.184, 0.20 and 0.411 gm. per 100 c.c., respectively.

No discussion of these reactions in kala-azar would be complete without pointing out that various flocculation reactions have been described for syphilis. In discussing the nature of these Wells (1925) states:

"As seen in the above reactions the capacity of syphilitic serum (and spinal fluid) to produce flocculation with various colloidal or crystalloid precipitating reagents, is distinctly increased over the normal. The flocculated material is generally found on examination to contain both the serum protein, especially globulin, and the other agent involved in the flocculation. When the latter is a lipoid it seems to be the predominating part of the precipitate. Whether this change in properties of the serum and spinal fluid depends merely on the increased proportion of globulins, or on changes in the quality of the globulins, or the presence of some new factor, possibly an antibody, is not known. That the mere increase of globulin cannot be responsible is indicated by the fact that many infections cause an increase in the proportion of globulin in the blood without causing it to give these reactions which are usually given by the serum of syphilitics, nor does the isolated globulin of normal serum give positive reactions. . . . Kolmer believes that the fundamental mechanism of complement fixation in syphilis is identical with these macroscopic colloidal flocculation reactions. . . . It is entirely reasonable to assume that in the Wassermann reaction the same antibody-like substance is operative as in

the various flocculation reactions; that in the former, flocculation occurs which is invisible to the naked eye, but sometimes visible microscopically by the dark-field illumination method. In a general manner the tissue extracts yielding best results in the complement-fixation reaction also prove most sensitive in the macroscopic flocculation reaction, e.g., the cholesterolized alcoholic extracts."

The fact that many tests for syphilis may be due to mechanisms similar to those in kala-azar would seem to lessen the use of the miscellaneous tests for the leishmaniosis. The literature indicates, however, that the flocculating tendency is so much stronger in kala-azar that there need be no confusion between the two diseases.

Also, if Kolmer's idea is correct that the Wassermann reaction in syphilis is dependent upon the same globulins as are precipitated in the flocculation reactions, there is evidence that the globulins appearing in kala-azar and syphilis are different because, as pointed out previously, kala-azar serums do not give positive Wassermann tests. Recently, Lloyd and Paul (1928) have adduced evidence that the formol-reacting mechanism in kala-azar is dependent upon two factors, one of which is specific to the disease and is associated with the euglobulin fraction, and the other of which is non-specific, is associated with the pseudoglobulin plus albumin fraction, and can be supplied from normal serum.

IV. *Trypanosomiasis*

In general a diagnosis in trypanosomiasis is made by finding the parasites in the blood or other material (such as gland juice in sleeping sickness, spinal fluid in the later stages of sleeping sickness or scrapings of the plaques in dourine) or by finding them in the blood of animals subinoculated from such sources. A reliable serological method would be of inestimable value in latent or low grade infections, but so far this has been achieved only with the complement fixation test in dourine (*Trypanosoma equiperdum*) of horses and related animals. Experimental work indicates, however, that antibodies do arise as a result of infections with other trypanosomes and that in these, too, serological tests may, presumably, be perfected.

1. COMPLEMENT FIXATION

The long list of investigations on complement fixation in trypanosomiasis can be roughly divided into three groups according to the test antigen used: (1) extracts of normal organs, thus making the

test more or less identical with the Wassermann reaction, (2) extracts of organs from animals infected with trypanosomes, and (3) extracts of isolated parasites.

The studies on complement fixation in trypanosomiasis with test antigens consisting of either aqueous or alcoholic extracts of *normal* organs attempted to secure non-specific fixation, such as is employed in the Wassermann test in syphilis. They indicate strongly that rabbits infected with certain strains of trypanosomes do give positive Wassermann or flocculation tests, but that other animals (including man) give at best irregular and entirely unsatisfactory reactions. In fact, in man, as has already been pointed out, in view of the refined techniques now in general use, it is probable that trypanosomiasis is not to be considered a disturbing factor in the Wassermann reaction for syphilis.

Among the investigations indicating that rabbits give positive Wassermann reactions, the following are of particular interest. The first workers, Landsteiner, Müller and Pötzl (1907) used aqueous extracts of normal guinea-pig liver in a study of complement fixation in forty-two rabbits, ten of which were infected with *T. equiperdum*. They found a much stronger fixation, on the average, after than before infection and in infected than in normals. Levaditi and Yamanouchi (1908), using probably the same type of test antigen, obtained negative results in two rabbits with *T. equiperdum,* but positive results in seven of nine animals infected with *T. brucei*. In the same year, Hartoch and Yakimoff (1908), with the same test antigen, found that the complement-binding property arose eleven to thirteen days after infection with *T. equiperdum* in seven rabbits. With alcoholic liver extracts of normal rabbits and guinea-pigs, Schilling and von Hösslin (1908) sometimes obtained good and sometimes unsatisfactory results with the serum of infected rats, rabbits, guinea-pigs, and oxen. Similarly, Manteufel and Woithe (1908), using both aqueous and alcoholic extracts of liver and blood of normal rabbits and guinea-pigs, reported contradictory results in rabbits, guinea-pigs and rats infected with *T. equiperdum* and *T. brucei*. Unsatisfactory results were also reported in rats infected with dourine by F. Blumenthal (1908). Although by no means invariable, a positive Wassermann reaction was found in rabbits by Browning and M'Kenzie (1910), (*T. brucei*); M'Intosh (1910), (*T. brucei*); F. Blumenthal (1911), (*T. equiperdum*); F. Meyer (1911), (*T. equiperdum*); Halberstaedter (1912), (*T. equiperdum*); and rarely by Braun and Teichmann (1912 c), (infected with *T. equinum* or immunized with *T. brucei*). Of particular interest in this connection

is the recent work of Landsteiner and van der Scheer (1927), who have shown that the serums of rabbits become strongly Wassermann positive when the animals are immunized with dead trypanosomes (*T. equiperdum*), but less regularly so when they are infected with different strains of the parasite. Parallel results were obtained with the Sachs-Georgi flocculation test. These authors also obtained complement fixation with alcoholic extracts of the parasites.

In all of this work on rabbits occasional uninfected animals gave positive Wassermann tests. This was accounted for by some workers as due to coccidiosis (see Schilling and von Hösslin, 1908, for a discussion of this possibility), but was denied by others. In any case, most investigators were careful to use only rabbits that were negative previous to infection.

In the literature just reviewed practically all laboratory animals have given unsatisfactory results in the Wassermann test. Similarly, Pavlosévici (1910) stated that although Wassermann "antibodies" may be demonstrated in laboratory animals, the method is useless in the diagnosis of dourine in stallions. Unsatisfactory results were found in sleeping sickness by Levaditi and Yamanouchi (1908), Bourret (1913) and Heckenroth and Blanchard (1913). The last observers, however, obtained positive reactions in over half of sixty-eight uncured patients with no observable signs of syphilis.

The early results on *specific* complement fixation in trypanosomiasis were, for the most part, contradictory. This was no doubt due to the fact that the test antigens consisted of extracts of organs from infected animals and did not contain a sufficiently high concentration of parasitic materials. Citron (1907, see Citron, 1909) obtained complement fixation with the serums of rabbits which had been injected with aqueous extracts of the blood and organs of rats infected with *T. brucei*. Suitable controls were employed with normal organ extracts. Inconsistent results were reported by della Vida (1907) who worked on rabbits and guinea-pigs infected with *T. brucei*, *T. equinum*, and *T. gambiense*, and used as test antigen saline extracts of dried blood containing trypanosomes; by Weber (1907) who used aqueous extracts of the organs of rats infected with *T. brucei*; by Manteufel (1908) who used aqueous extracts of blood and organs of rats infected with *T. lewisi* and *T. equiperdum*; by Manteufel and Woithe (1908) who used alcoholic and aqueous extracts of animals infected with *T. equiperdum* and *T. brucei* (as well as extracts of normal organs, as has been previously noted) and by Schilling and von Hösslin (1908) who worked on infected rabbits, guinea-pigs, rats and oxen and used alcoholic extracts of livers from

animals infected with *T. brucei* and *T. equiperdum*. The latter, however, sometimes obtained very good results.

With essentially the same test antigens (aqueous extracts of livers of guinea-pigs infected with *T. equiperdum, T. brucei, T. evansi* and *T. equinum*), consistently positive results were reported by Hartoch and Yakimoff (1908) in seven rabbits infected with *T. equiperdum,* and negative tests in two control rabbits and in the experimental ones before infection. It is to be recalled that the same authors obtained practically the same desirable results when aqueous extracts of normal livers were used. Somewhat later, inconsistent results were again obtained by Beck (1910) in infected rabbits and guinea-pigs when tested with carbolized saline extracts of livers of either normal or infected laboratory animals, and Zwick and Fischer (1910) came to the conclusion that neither agglutination nor complement fixation (using as test antigen carbolized saline extracts of the liver, heart, and spleen of horses and rats infected with *T. equiperdum*) could be used for diagnosis in dourine.

Levaditi and Mutermilch (1909) maintained that the inconstant results of most of the former investigators on specific fixation were due to faulty technique and in particular to the use of test antigens containing, besides trypanosome material, lipoidal organ extracts which led to non-specific fixation. Consequently, they prepared their test antigens from trypanosomes freed from blood by two centrifugations, dried *in vacuo* over sulphuric acid and powdered. For use 0.05 gm. of this powder was extracted in 4 or 5 c.c. of saline at 37°C. for one hour. Later, they used simple suspensions of the trypanosomes in saline. Their antiserums were obtained from guinea-pigs after immunization with the trypanosome extract or during the course of infection. Working with *T. evansi* and several strains of *T. brucei*, they obtained constant results and concluded that the complement fixation reaction can be used to detect infections and is specific for the genus but not for the species. M'Intosh (1910) in parallel tests with a Wassermann test antigen and one consisting of a saline emulsion of isolated trypanosomes found that the latter (specific complement fixation) gave stronger reactions.

The sum total of the published work through 1910 indicated that under controlled laboratory conditions complement-fixing antibodies could be demonstrated in trypanosomiasis; but there was little to indicate that it could be made a practical method of diagnosis. From 1911 onward, however, the test was perfected and used extensively for the diagnosis of *T. equiperdum* (dourine) in horses and mules.

Winkler and Wyschelessky (1911) obtained positive fixation tests

with the serums of fifteen horses having dourine, and negative tests with the serums of twenty-five normal horses by using as test antigen the clear supernatant after the following process: one part of washed *T. brucei* or *T. equiperdum* to ten to twenty parts saline, shaken one to three days with glass beads, centrifuged, and filtered through a Berkefeld candle. This was followed in 1913 by the monumental work of Mohler, Eichhorn and Buck who ran tests on 8,657 horses in Montana, 1,076 of which gave positive reactions. The authors felt that many latent cases were thereby diagnosed which would otherwise have escaped detection. Their actual technique was the same as that developed by Mohler and Eichhorn (1911) for the diagnosis of glanders. They used filtered saline extracts of fresh macerated spleens (1 per 40 c.c. saline) from rats just dead of an infection with *T. evansi*, as well as saline extracts of washed concentrated trypanosomes; in preparing these they used saponin to lake the blood-cells. Watson (1914) made extensive use of their method in Canada and by 1914 had carried out 3,200 fixation tests, using spleens from infected rats. Later (1915 and 1920), he used an aqueous extract of isolated trypanosomes, the technique of which is described in detail at the end of this section. After his careful work, he considered the test certain and specific in diagnosing the disease, and often positive before symptoms were apparent or during latent stages. Furthermore, although in practice it might become positive by eleven days after infection, no animal was considered free of the disease unless negative two months after last exposure. With extracts of infected spleen Wehrbein (1914) found 10 per cent of 5,000 tests positive (two tests for each animal). Following Levaditi and Mutermilch's technique, Waldman and Knuth (1920) reported consistent results in sick and healthy horses.

The suitability of alcoholic and carbolized saline extracts of concentrated *T. equiperdum* from rats was considered in detail by Dahmen (1922). In testing 1,324 horses, both extracts gave specific fixation, but the alcoholic was five to ten times more active. Furthermore, the two extracts seemed to owe their activity to different components because when the trypanosomes were extracted first with saline, then with alcohol, both extracts were active, but the alcoholic was the more so. The aqueous test antigen was prepared by washing and concentrating the trypanosomes by centrifugation, adding 0.5 per cent carbolic acid in saline (1 c.c. per rat), shaking vigorously two days, placing on ice one day and using the supernatant. In making the alcoholic extract, the concentrated trypanosomes were dried at 50°C., pulverized in a mortar and 0.1 gm. of powder plus 1.0

c.c. of ether was shaken an hour and the residue after filtration was allowed to stand in 1.0 c.c. of absolute alcohol two days at 37°C., after which the colorless supernatant (obtained by centrifugation for three minutes at 3,000 r.p.m.) was diluted drop by drop with five to ten volumes of saline. No fixation was obtained with organ extracts unless the organs were infected, nor with the specific antigen when tested against the serums of horses having various maladies other than dourine, thus indicating the specificity of the reaction.

Marcone and de Gaspari (1921), Nolte (1921), Nusshang (1921), and Robinson (1926) have all recognized the utility of the complement fixation reaction for the practical diagnosis of dourine.

In a series of communications Bessemans (1921, 1921 b, 1922, 1922 b) and Bessemans and Leynen (1922 and 1923) have found the reaction to be of great value in that it is positive in clinically positive animals, and sometimes even precedes recognizable symptoms by two months or more, persisting in those animals which have once given a positive reaction and being negative in those animals in which no grounds could be found for suspecting infection. (See Bessemans and Leynen, 1923.) Bessemans (1921) found that the non-specific fixation of complement by normal horse serum was greatly reduced by heating at 56-58°C. for thirty minutes and was destroyed by heating at 60°C. for thirty minutes; he, therefore, advocated the use of the latter method to avoid false positives. Later he (1922 b) investigated the anticomplementary and antigenic efficiency of some of the different methods of preparing test antigens, with the results recorded in Table 9, from which it can be seen that the only practical methods are the last two. Furthermore, the aqueous solvents of the concentrated trypanosomes seemed more reliable than the alcoholic. Bessemans and Leynen (1922), using the Mohler-Reynolds method of obtaining trypanosomes and preserving them in Watson's or Mohler's fluid, have studied the comparative efficacy of *T. evansi, T. brucei, T. equiperdum, T. rhodesiense* and *T. lewisi* as sources of test antigen and have found that the first three are superior to the last two. Furthermore, when the trypanosomes were obtained from guinea-pigs, it was best to use them before the first crisis. (See also page 118.)

In a very intensive study of one stallion infected with dourine, Schoening and Formad (1923) found complement fixation to be stronger in the spinal fluid than in the blood serum taken at the same time.

Not all of the recent studies on dourine are as satisfactory as those reviewed. Thus, Balozet (1924), after trying a number of

TABLE 9

ANTIGENIC AND ANTICOMPLEMENTARY POTENCY OF DIFFEPENT TEST ANTIGENS IN THE COMPLEMENT FIXATION TEST FOR DOURINE (FROM BESSEMANS, 1922 B)

Test antigens	*Anticomplementary action*	In vitro *antigenic action*
Aqueous or alcoholic extracts of infected organs	Very strong	Weak and variable depending upon the quantity of parasites in the organ
Supernatant of heavily infected rat blood after sedimentation of blood-cells	Strong	Weak or *nil*
Extracts prepared by treating infected blood with ether or ligroin, separation into three layers, preservation and washing of middle layer and elimination of volatile material	Weak	Very weak
Emulsions of trypanosomes separated by centrifugation and diluted in the alcoholic or the aqueous fluids of Mohler or Watson	Weak	Intense
Emulsions of the sediment obtained by centrifuging infected blood after laking blood-cells with 0.5 per cent saponin. (Mohler-Reynolds)	High (due to presence of cell debris)	Very intense

different test antigens, found none that yielded clearly positive results, and Barotte and the same author (1924) felt that the utility of Watson's technique for the disease in the donkey required further investigation. In a later paper, however, Barotte (1926) came to the conclusion that the doubtful reactions in donkeys and mules were due to insufficient preliminary heating of the serum, and that such serums must be heated at 60-62° C. for at least thirty minutes to destroy their anticomplementary properties.

After trying to use complement fixation for the diagnosis of mal de caderas (*T. equinum*), Quiroga (1924) concluded that it could not replace existing methods of diagnosis.

Besides the work on complement fixation as a practical method of diagnosing dourine, there have been a series of recent investigations demonstrating the practicability of using the reaction in experimentally infected animals to ascertain just when antibodies are first demonstrable. In the early work of Hartoch and Yakimoff (1908),

which has been reviewed on page 53, complement-fixing antibodies were demonstrable in the blood of rabbits eleven to thirteen days after infection with *T. equiperdum, T. evansi, T. equinum,* and *T. brucei.* Braun and Teichmann (1912 c), in their study of immunization against trypanosomes, obtained positive fixation in rabbits infected or immunized with *T. equiperdum, T. brucei* or *T. equinum,* in horses infected with *T. equiperdum* and in cattle infected with *T. brucei,* with a carbolized saline extract of washed trypanosomes isolated from infected rats. Furthermore, they verified the findings of all previous investigators that the reaction was only suitable to diagnose the presence of the genus *Trypanosoma* and not the particular species. In collecting the parasites the animals were bled into a mixture of sodium citrate and normal horse serum or antiserum against rat red cells which agglutinated the red cells; then the trypanosomes from the supernatant were concentrated and washed by centrifugation. Positive reactions appeared in the serums of ten rabbits, eight to nine days after infection with *T. equiperdum,* according to Offermann (1915) who used saline extracts of washed parasites, and also in seven similarly infected dogs, according to Woods and Morris (1918), who used saline extracts of infected spleens of rats. Moreover, the serum remained reactive for months when kept in sealed tubes (Offermann, 1915). In a thorough study of the reaction in rabbits, Chu-Jen Ku (1926) tried several test antigens and found the most satisfactory to be Dahmen's alcoholic trypanosome extract. From ten rats, 4 c.c. of concentrated trypanosomes (sp.?) were obtained and diluted for use at the rate of 0.005 c.c. of alcoholic extract to 1.0 c.c. of saline. Complement fixation tests on forty-two samples of serums from fifteen normal rabbits were uniformly negative, on seventeen samples of serums from two infected rabbits with pronounced secondary symptoms were uniformly positive, and on five infected rabbits at two to three day intervals were negative for the first thirteen to fourteen days of the infection, then became positive and remained positive throughout the remainder of the infection (three gave a stronger titer than the other two). The change from negative to positive appeared almost simultaneously with the appearance of secondary symptoms. Positive reactions with normal rabbits (a difficulty encountered by many previous investigators) were ruled out by the dilution of the antigen noted above.

Mention should here be made of a short paper without protocols or experimental details by Lanfranchi (1912 b), in which the usual procedure was reversed and the serums from infected animals (*T. brucei* or *T. equiperdum*) were tested for antigen instead of anti-

body. Thus, their serums were mixed with the serums of dogs highly immunized (by a special technique not given) with *T. brucei.* The homologous species with *T. brucei* antiserum fixed complement throughout the whole course of infection in the guinea-pig, when trypanosomes were either numerous or absent; the heterologous species also gave positive fixation, but was more clearly defined toward the end of the infection. Although not discussed by Lanfranchi, it would seem that he is dealing with a true antigen-antibody reaction in which the antibody is contained in the hyperimmune dog serum and the antigen consists of the trypanosomes or their products in the serum from the infected animals. This is borne out especially in the precipitation work where positive results were obtained when numerous parasites were in the blood and negative results were obtained during the periods of trypanolytic crises.

Some serological method is especially needed in South American trypanosomiasis (Chagas' disease) because the parasites are very difficult to demonstrate after the onset of the so-called chronic stage. The complement fixation reaction seems promising provided the diagnoses of the disease by the various investigators can be accepted. * The first workers, Guerreiro and Machado (1913) found that aqueous extracts of the spleen of highly infected puppies, containing many leishmaniform parasites, were superior to various alcoholic and aqueous extracts of trypanosomes as test antigens. Such spleens were triturated in 1 per cent phenol, the filtrate added to twice its bulk of 1.7 per cent saline and the subsequent filtrate used as test antigen. They considered the reaction specific and not correlated with the Wassermann reaction, in agreement with all subsequent workers. Furthermore, in a personal communication to Villela and Bicalho (1923), they reported conclusive results in a study of the serums and spinal fluids from eighty persons most of whom had the chronic disease. Villela and da Cunha, in unpublished work of 1919 and 1920 according to Villela and Bicalho (1923), after working on eighteen serums of both positive and negative cases with various aqueous extracts of infected and normal organs of dogs and guinea-pigs, concluded that the reaction only took place in the presence of infected organs and in proportion to the number of parasites. The hearts of puppies were particularly rich in parasites, and hence, in complement-fixing properties. Villela and Bicalho (1923) attempted to control the great variability of the test antigens prepared from infected organs. The most promising of these, i.e., the heart and spleen, were used

* In many cases the criteria used to establish the presence of the disease seem insufficient.

together. After removal, aseptically, from a highly infected puppy, they were freed of blood clots, washed in saline, cut in small pieces, and triturated in a mortar; to one part of pulp were added distilled water two parts, glycerin one part, and phenol to make a final concentration of 0.5 per cent. After forty-eight hours at room temperature, the mixture was strained through gauze and allowed to stand in the refrigerator until a deposit formed; then the filtrate was kept on ice ready for use. In this form it retained its activity for four months; in fact, the preservation seemed to diminish the anticomplementary activity. The results were quite striking: in thirty-three tests upon the serums and six upon spinal fluids from patients with Chagas' disease, only three of the former and two of the latter gave negative results; in eleven tests on normal subjects, all were negative, as were all those with test antigens prepared from normal dogs. In reviewing the tests made by the different workers, Villela and Bicalho found that in sixty-seven reactions (both on serums and spinal fluids of patients) only eight were negative, and these, the evidence indicated, occurred in the acute and not the chronic stages of the disease.

Recently, Lacorte (1927), using Villela and Bicalho's technique, found that his test antigens did not keep very well and that the spleen alone was a superior source of material. Of 200 cases of suspected Chagas' disease, 159 yielded positive reactions while sixteen normal persons, two malarial patients and three persons with ulcers, influenza, and rheumatism gave negative reactions.

A comparison of the so-called conglutination test with complement fixation was carried out on serums from nineteen horses infected with dourine and thirty normals, by Wehrbein (1915). With a single exception the results were similar, but he believed that the conglutination method was more difficult to use and more likely to suffer from faulty technique.

Among the many variations in technique that have been developed for the study of specific complement fixation in dourine of horses and related animals, I have selected that of Watson because he used isolated trypanosomes for his test antigen. The technique is given in detail in a paper of 1915, and the present summary is taken from one of 1920.

A stock test antigen is prepared from trypanosomes obtained from white rats killed at the height of their infection with *T. equiperdum* and bled into about an equal quantity of citrated salt solution. The mixture is strained through a double layer of gauze to remove small clots and centrifuged for four to five minutes at 1,500 r.p.m. in

TABLE 10

PRELIMINARY TITRATION TO DETERMINE THE ANTIGENIC POWER OF THE TEST ANTIGEN IN THE COMPLEMENT FIXATION TEST FOR DOURINE (REARRANGED FROM WATSON, 1920)

Tubes	*Saline 0.85%*	*Inactivated serum* †	*Antigen 1: 20*	*Complement (dilution by previous titration)*		*Sensitized cells*		*Results*
	c.c.	c.c. Known positive	c.c.	c.c.	Mix and incubate 1 hr. 10 min. at 38-39° C.	c.c.	Mix: Water bath 2 hrs.	
1	1.0	0.1	0.02	0.5		1.0		No hemolysis (fixation) in at least some tubes
2	1.0	0.1	0.05	0.5		1.0		
3	1.0	0.1	0.1	0.5		1.0		
4	1.0	0.1	0.15	0.5		1.0		
5	1.0	0.1	0.2	0.5		1.0		
6	1.0	0.1	0.25	0.5		1.0		
7	1.0	0.1	0.3	0.5		1.0		
8 C*	1.0	0.1	0	0.5		1.0		Hemolysis
		Known negative						
9	1.0	0.2	0.05	0.5		1.0		Hemolysis (no fixation)
10	1.0	0.2	0.1	0.5		1.0		
11	1.0	0.2	0.2	0.5		1.0		
12	1.0	0.2	0.3	0.5		1.0		
13	1.0	0.2	0.4	0.5		1.0		
14	1.0	0.2	0.5	0.5		1.0		
15	1.0	0.2	0.6	0.5		1.0		
16 C	1.0	0.2	0	0.5		1.0		Hemolysis
17 C	1.0	0	0.25	0.5		1.0		
18 C	1.5	0	0	0.5		1.0		
19 C	2.0	0	0	0.5		1.0		No hemolysis

* C = Control.

† Horse serum has to be inactivated for 30 minutes at 58° C. Mule serum has to be inactivated for 30 minutes at 62° C.

small 10-c.c. centrifuge tubes. Several layers can then be separated: (1) a cloudy supernatant containing many parasites, (2) an upper layer of blood-cells mixed with trypanosomes, and (3) a layer mainly of red cells. From layers 2 and 3, many trypanosomes can be recovered by adding more citrated saline and recentrifuging. Eventually, the trypanosomes in the supernatant layers are washed in 0.85 per cent saline, centrifuged, and obtained in a pure white concentrated mass. Under optimum conditions ten rats will yield 5.0 c.c. of parasites which will make 100 c.c. of test antigen or enough for over 500 diagnostic tests. The trypanosomes are then placed in twice

TABLE II

METHOD OF PROCEDURE FOR A DIAGNOSTIC COMPLEMENT FIXATION TEST IN DOURINE (WATSON, 1920)

	Tube No.	*Salt solution*	*Test* serum*	*Antigen*	*Complement †*	*Mixture of hemolytic serum and red cells ‡*	*Fixation §*
		c.c.	c.c.	c.c.	c.c.	c.c.	
Suspected serum	1	1.0	0.2	0.2	0.5	1.0	++++ ‖
	2	1.0	0.15	0.2	0.5	1.0	++++ ‖
	3	1.0	0.1	0.2	0.5	1.0	++++ ‖
Serum control	4	1.0	0.2	0	0.5	1.0	— ‖
Known positive serum	1	1.0	0.2	0.2	0.5	1.0	++++
	2	1.0	0.1	0.2	0.5	1.0	++++
Serum control	3	1.0	0.2	0	0.5	1.0	—
Known negative serum	1	1.0	0.2	0.2	0.5	1.0	—
Serum control	2	1.0	0.2	0	0.5	1.0	—
Reagent controls:							
(a) Antigen	1	1.0	0	0.2	0.5	1.0	—
(b) Hemolytic sys.	2	1.0	0	0	0.5	1.0	—
(c) Hemolytic ser.	3	1.5	0	0	0	1.0 Red cells only	..
(d) Complement	4	1.5	0	0	0.5	0.5	..
(e) Red cells	5	2.0	0	0	0	0.5	..

* Horse serum is inactivated by heating for 30 minutes at 59° C.; donkey and mule for 30 minutes at 62° C.

† One unit per 1 c.c. [One-half amounts are used throughout.]

‡ The same mixture used for titration of antigen. It is added after the first ingredients are incubated at 38-39° C. for 70 minutes.

§ The first reading is made after a second incubation of 2 hours; the second after 12 hours at cool room temperature: ++++ signifies fixation; — hemolysis; .. no hemolysis.

‖ A strong positive. A weak positive would yield readings of +++, ++, +, — in the 4 tubes, respectively.

their volume of preserving fluid (0.85 per cent NaCl. 90 parts; pure neutral glycerin, 10 parts; and formalin, 0.1 part) and stored on ice in 1 c.c. sealed amber ampoules. In attempting to improve Watson's technique, Reynolds and Schoening (1918) centrifuged the strained blood for twenty minutes at 2,000 r.p.m., so that a deposit of trypanosomes and blood-cells was obtained which was agitated for twenty minutes with distilled water to lake the blood cells, cen-

trifuged for thirty minutes and the trypanosomes subsequently washed and concentrated in saline. The distilled water had no detrimental effect on the antigenic property of the trypanosomes and diminished their anticomplementary action.

In titrating his antisheep rabbit serum, he defined a unit of antiserum as the amount necessary to dissolve 1 c.c. of 4 per cent sheep corpuscles in the presence of 1 c.c. of a 1:20 dilution of fresh complement, instead of the usual 1 c.c. of 5 per cent cells and 1 c.c. of 1:10 dilution of complement (see page 20). Similarly, a unit of complement is the amount necessary to dissolve 1 c.c. of 4 per cent cell suspension in the presence of two units of hemolytic serum. The requisite titers are obtained by preliminary titrations (1) of antiserum using dilutions from 1:400 through 1:5,000 in 1 c.c. amounts with 1 c.c. of 1:20 complement and 1 c.c. of 1:25 cells, and (2) of complement with dilutions of 1:20 through 1:60. Then the antigen is tested against a known positive and negative horse serum using one half amounts. (See Table 10.) The amount of antigen to be selected as the titer for the final tests is that which gives complete fixation with the positive serum while double the quantity with the negative serum does not inhibit hemolysis. The actual test is then made according to Table 11.

2. AGGLUTINATION *

Agglutination in trypanosomes was probably noted first in *T. lewisi* in 1900 by Laveran and Mesnil (1900 and 1900 b) and was described in detail by them in 1901. Since then it has been reported by numerous observers and studied in more or less detail by several. (See Jürgens, 1902; Francis, 1903; von Prowazek, 1905; Düring, 1908, and Manteufel, 1908). An interesting summary of the early work has been made by Laveran and Mesnil (1912). The most striking feature of the process is that the individual trypanosomes in the agglutinated masses exhibit a definite orientation of their bodies, which is clearly indicated in stained slides. At the beginning of the process two trypanosomes adhere by their posterior ends (Figure 1, 1). Later, large masses are formed with the same orientation, i.e., the posterior ends are in the center (Figure 1, 2). In connection with this mode of orientation, Laveran and Mesnil pointed out the interesting distinction between agglutination, where

* Some authors prefer to use the term *agglomeration* in trypanosomes to differentiate the process from agglutination in bacteria. At the present stage of knowledge this seems scarcely necessary. For a discussion of the subject see Schindera (1922).

the parasites are passive and attached by the posterior end, and fixation of the parasites in such localities as the intestine of the insect hosts, where they play an active rôle and attach themselves by their anterior ends. These investigators further found that serums which agglutinated *T. lewisi* when living also agglutinated them when killed with formalin or chloroform vapor, and *vice versa*. The killed trypanosomes, however, showed no orientation of their bodies, but were arranged pell-mell. Manteufel (1908), on the other hand, found that when trypanosomes were killed by heating to 45° C., they were not agglutinated by specific serums.

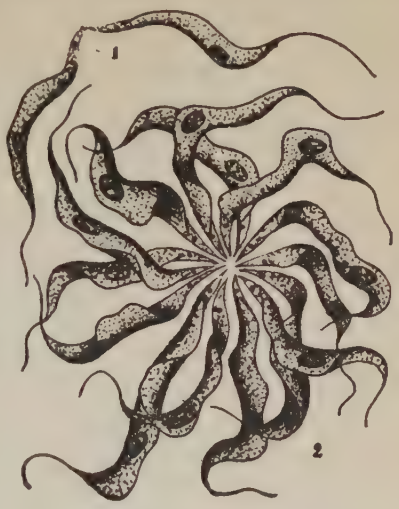


FIG. 1.—Agglutination of *Trypanosoma lewisi* showing the orientation of the parasites as seen in stained preparations (x about 1300). 1, two organisms attached by their posterior ends; 2, a primary agglutination rosette consisting of fourteen individuals. (From Laveran and Mesnil, 1912.)

Serum from normal rats does not agglutinate *T. lewisi*. During the course of an infection, it acquires a weak titer which increases at the termination of an infection and is generally highest of all in hyperimmunized animals (i.e., rats receiving a number of injections of *T. lewisi*). At no time, however, is the titer very high. Thus, Laveran and Mesnil reported it as varying from 1:5 to 1:50 and Francis (1903) noted one case of 1:200. Some time after infection or immunization the titer gradually falls.

In agglutination among the pathogenic trypanosomes (Figure 2), the same orientation is observable as was noted in *T. lewisi*. Lignières (1903) reported that serums from cattle, rats, cats and humans agglutinated *T. equinum* slightly, while those of sheep, pigs, rabbits and horses agglutinated it strongly, especially if the animals from which the serum was taken were infected. On the contrary, normal serums from frogs, chickens, guinea-pigs, and dogs seemed ineffective. Franke (1905), working with the same species, noted the agglutinating power of the serum of a monkey, cured of its infection, when tested with the homologous trypanosome. Zwick and Fischer (1910), on the other hand, in working on dourine, obtained negative results

and concluded that neither complement fixation nor agglutination was a suitable method of diagnosis.

Beginning with 1911 a series of papers clearly indicated that with the proper technique agglutinins in high titers could be demonstrated in animals infected or immunized with pathogenic trypanosomes. Thus, Lange (1911), working with *T. equiperdum, T. brucei,*

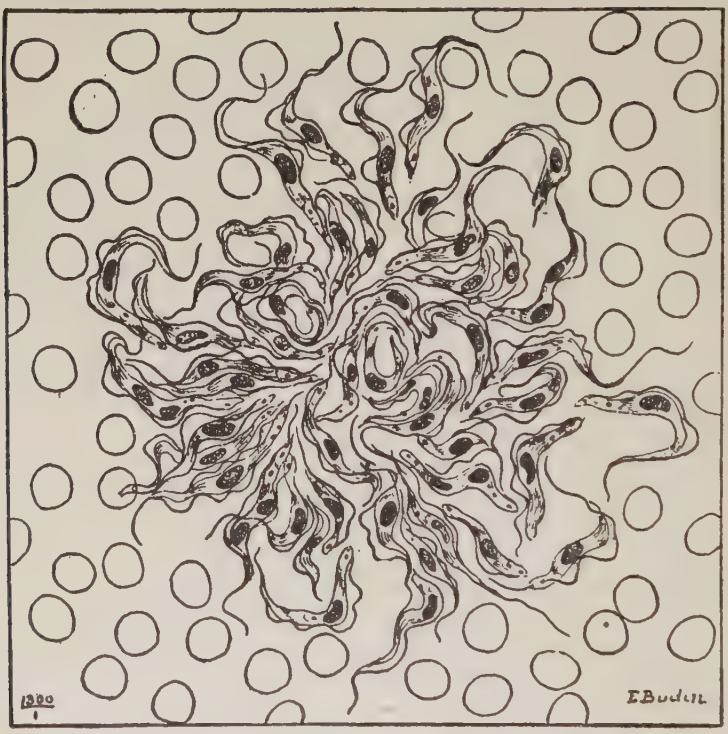


Fig. 2.—Agglutination of *Trypanosoma brucei* under the action of immune serum. Stained preparation showing approximately the same orientation as in Fig. 1 (x 1300). (From Ponselle, 1923.)

and *T. gambiense,* ascertained that the serums of normal animals were generally negative and never agglutinated the organisms in dilutions higher than 1 : 100, whereas those from animals immunized with dead trypanosomes showed titers as high as 1 : 1,600 and those from infected animals might reach 1 : 12,500. His test antigen consisted of washed isolated trypanosomes suspended in saline and preserved with formalin. In carrying out the tests equal parts of anti-

serum and test antigen (0.2 c.c.) were mixed and incubated six to twelve hours at 37° C., then at room temperature for varying lengths of time (in doubtful cases twenty to twenty-four hours). This author also reported the existence of group reactions, but the homologous were always higher than the heterologous reactions.

Winkler and Wyschelessky (1911) indicated that the method might be of considerable value for the diagnosis of dourine. With a test antigen consisting of a saline suspension of isolated washed trypanosomes, the serums of fifteen dourine horses gave titers ranging from 1:800 to 1:20,000, with the majority of them between 1:8,000 and 1:10,000. In marked contrast the serums of fifty normal horses gave the following results: thirty-one showed no agglutinins, seventeen gave a titer of 1:20 and two a titer of 1:50. Besides this work the authors also studied agglutination of *T. equiperdum* and *T. brucei* in infected dogs, sheep, and rabbits. Their serums showed group reactions. Comparative results of complement fixation, precipitation, and agglutination in ten horses showed a very good correspondence. Mattes (1912) studied the agglutinating property of normal and immune serums upon *T. gambiense, brucei, equiperdum,* and *congolense.* The test antigen consisted of saline suspensions of isolated washed organisms which could be kept for a week by preserving with 0.3-0.6 per cent formaldehyde. Normal serums never caused agglutination in dilutions over 1:100; those of the horse and ox showed the highest titers, whereas those of man, sheep, goat, pig, rabbit, guinea-pig, and mouse never reacted in dilutions higher than 1:20. Animals were immunized by injecting, first, dead or avirulent, and then, after about a week, virulent organisms. Serums from these showed a somewhat rapid elevation of titer, but were not rigidly specific, although they showed a higher titer for homologous than for heterologous strains. For example, immune serum from a rabbit infected with *T. brucei* agglutinated *T. brucei* in dilutions of 1:3,000, *T. equiperdum* in 1:2,000, and *T. congolense* and *T. gambiense* in 1:1,500. Agglutinins were not destroyed by heating several hours at 60° C. and were found in exudates from serous cavities. A summary of his more important results is given in Table 36. Ruppert (1912) with a similar test antigen found that whereas normal serum may agglutinate up to 1:200, the serums of three horses (one with *T. equiperdum* and two with *T. brucei*) agglutinated in dilutions of 1:5,000. After immunizing dogs against *T. brucei,* by a special method which is not described, Lanfranchi (1912 b) found that the serums of such animals agglutinated the homologous species in dilutions up to 1:75,000 and *T. equiperdum* from 1:50,000 to 1:60,000.

Comparing the relative value of complement fixation and agglutination as a means of diagnosis, Offermann (1915) concluded that although both are valuable, the former is superior. This investigator worked with rabbits infected with *T. equiperdum* and his results are very interesting in showing when the antibodies arise during the course of infection. Thus, the serums from ten rabbits showed no agglutinating property before infection, but after infection was first demonstrated as follows: in three on the sixth day, in one on the thirteenth, in two on the fourteenth, in two on the sixteenth, in one on the nineteenth, and in one on the twenty-third. Complement fixation appeared somewhat earlier in the serums of these animals—generally from eight to ten days after infection. Recently Marcone and de Gaspari (1921) found that normal horse serum agglutinated *T. equiperdum* in dilutions up to 1:200, whereas serum from infected horses gave titers of 1:20,000. Dahmen and David (1921) and Dahmen (1922) considered the reaction of value in the diagnosis of dourine.

3. PRECIPITIN TESTS

Just as in the complement fixation studies in trypanosomiasis, the most extensive work on precipitins has been concerned with attempts to devise a diagnostic test for dourine. Precipitins were probably first studied in trypanosomiasis by Mayer (1905) who used aqueous test antigens. The filtrate from *T. brucei* after autolysis in saline at 37° C. for 48 hours was non-reactive, but the filtrate derived from trypanosomes which had been digested for a week with trypsin was efficient. As early as 1908, Uhlenhuth and Woithe obtained positive results in dogs and horses with chronic infections of *T. equiperdum* after atoxyl treatment, but negative results in infected rabbits. From this they concluded that the test did not give promise of being an aid in diagnosis any more than did complement fixation in which Manteufel and Woithe (1908) had previously obtained contradictory results.

The first successful work with dourine infections was done by Winkler and Wyschelessky (1911), who used the same test antigen as in their complement fixation work. Ring tests on serums from fifteen infected horses were positive, whereas similar tests on twenty-five uninfected animals were negative. In positive serums a precipitate appeared at the interphase very quickly; in most cases immediately and in practically all cases within ten minutes. In negative serums a precipitate also appeared, but was fainter, appeared much later, and did not last as long. Comparative results with the precipitin,

complement-fixing and agglutinin reactions with serums from ten horses showed a good correspondence. Similar precipitin results were reported by Ruppert (1912) who used the same technique with *T. brucei* and *T. equiperdum;* he did not give details as to the number of animals studied.

In conjunction with his complement fixation studies where the serums of infected animals were tested for antigen instead of antibody, Lanfranchi (1912) also carried out some work on the precipitin test and found that by putting three parts of serum from infected guinea-pigs in contact with one part of hyperimmune (*T. brucei*) serum, the homologous species reacted throughout the whole course of infection, whereas the heterologous species (*T. equiperdum*) only reacted when the parasites were particularly numerous.

After the lapse of several years, there was a renewed interest in precipitins. Marcone and de Gaspari (1921) reported positive precipitin tests (ring tests) as well as complement fixation with extracts of *T. brucei* in six dourine horses, and negative results in uninfected animals. The infected horses, furthermore, gave negative Sachs-Georgi flocculation tests with an alcoholic extract of horse heart.

In an extensive study of the serums of 1,324 horses, Dahmen (1921 b and 1922) concluded that with aqueous extracts, the precipitin test was inferior to complement fixation, but that precipitin tests with alcoholic extracts (lipoid precipitation) were superior to complement fixation. The alcoholic extract was prepared by drying isolated washed trypanosomes at 50° C., pulverizing in a mortar, adding 0.1 gm. of powder to 1.0 c.c. of ether, shaking one hour, filtering, drying the filtered residue at 37° C., adding 1 c.c. of absolute alcohol, extracting two days at 37° C., centrifuging at 3,000 r.p.m. for three minutes and using the colorless supernatant diluted with physiological saline as test antigen. Using Dahmen's so-called lipoid precipitation on the serums of 1,016 horses, Fuest (1922) concluded that it was not only a practical aid in the diagnosis of dourine, but was more sensitive than complement fixation. For example, of the serums from 1,016 horses, eighty-three reacted positively in the precipitin test and seventy-three in complement fixation; from fifty-six known infected horses, fifty-three reacted positively in the precipitin test and forty in complement fixation. Table 12, which is rearranged from Fuest's data, shows a correlation between the two tests in 100 horses. This table is interesting in that it shows that there are the same number of positive tests with each method (complement fixation 23 ++++, 12+++, 9 ++, 22 +; precipitin 41 ++++, 25 ++), and yet inspection of the table indicates that,

TABLE 12

CORRELATION BETWEEN COMPLEMENT FIXATION AND DAHMEN'S PRECIPITIN TESTS (ALCOHOLIC EXTRACTS OF TRYPANOSOMES) IN 100 HORSES INFECTED WITH T. EQUIPERDUM (DATA FROM FUEST, 1927)

Precipitation	*Complement fixation* ++++	+++	++	+	?	—	Totals
++++	17	6	4	5	2	7	41
++	2	4	1	7	1	10	25
?	2	2	1	0	3	9	17
—	2	0	3	10	2	0	17
Totals	23	12	9	22	8	26	100

statistically, there is very little correlation. Such results might indicate the advisability of using both tests instead of one. In carrying out the tests, Fuest ruled out pseudo-positives by preliminary titrations, then overlaid 0.2 c.c. of serum in a small Uhlenhuth tube with 0.3 c.c. of a 2 per cent solution (generally) of the alcoholic trypanosome extract, incubated the tubes at 37° C. for one hour, read, left at room temperature another hour and reread. Control tests were carried out with each serum with a 4 per cent alcoholic extract of normal horse heart.

Dahmen (1920) also used a "lipoid fixation" reaction which he (1922) considered also superior to complement fixation. This test is a modification of one of the colloidal precipitation tests for syphilis devised by Meinicke on the hypothesis that the colloids in alcoholic extracts of tissues disturb the isotonicity of saline solutions and allow the combination of serum globulins and lipoids. Mixtures of the colloidal lipoid test antigen with syphilitic serums produce a precipitate which is less soluble in saline than the precipitate forming from a mixture of the same test antigen and normal serums. In Dahmen's test, however, a specific test antigen was used, and nonspecific test antigens did not give the reaction. He (1920) prepared this test antigen for lipoid fixation by adding an alcoholic extract of horse heart to half its volume of distilled water, allowing it to stand for two hours, adding 7 volumes (of the original extract) of distilled water and finally to this mixture adding his regular trypanosome test antigen in the same proportion as is used in the regular complement fixation test. Control test antigens were prepared in the same manner, except that other extracts, such as bacterial extracts,

were substituted for the trypanosome material. The actual test was carried out by adding 0.2 c.c. of the suspected serum to 1.0 c.c. of the horse-heart trypanosome-extract mixture, incubating sixteen to twenty-four hours at 37° C., carefully shaking the clumps that had flocculated out, until they were distributed throughout the tubes, adding 1 c.c. of 3 per cent NaCl to each tube and then, without shaking, incubating another hour at 37° C. In reading the tests, the clumps remained where serums were positive and did not break down on shaking, whereas they disappeared when serums were negative or when control test antigens were used. In the case of dourine Robinson (1926) also obtained positive results with this method, but withheld judgment as to its value compared with that of complement fixation.

4. AUTO-AGGLUTINATION OF RED CELLS

Normal blood-corpuscles in a cover-glass preparation tend to form rouleaux. These chains readily intertwine and anastomose to form various-sized masses, but the rouleaux formation in the main is not lost. Agglutinated red cells, on the other hand, do not form rouleaux, but tend to clump in islets of varying size and shape. The different appearance is readily observed microscopically and even macroscopically. Kanthack, Durham and Blandford (1898) first observed auto-agglutination of blood-cells of animals infected with *T. brucei* and also of normal cells upon the addition of serum of infected animals of the same species. Since then many investigators have confirmed its occurrence in the various trypanosomiases. Christy (1904), Thomas and Breinl (1905), Dutton and Todd (1905) and Martin, Leboeuf and Roubaud (1909) noticed it in fresh preparations from patients suffering with sleeping sickness. Later, however, Todd (1910) found that it occurred frequently but not invariably, while Beck (1910) observed its presence one day and absence another, and Werner (1913) described an acute case which ran its course without showing the phenomenon. Thomas and Breinl (1905), Dubois (1912) and others found it more common in untreated than in treated cases.

Yorke (1911) made a thorough study of the essential factors. He found that the phenomenon was absent (occasionally weakly present) at 37° C., well marked at 18-21° C., with an optimum at 0° C., and that it was reversible; that auto-agglutinins existed to a small extent in the blood of many normal animals, but to a greater extent in animals infected with trypanosomes; that the auto-agglutinins could be removed from plasma by absorption with erythrocytes

of the same animals at 0° C. and were active against cells of many animals of different species. He believed that the phenomenon was due to the enrichment of the normally occurring auto-, iso-, and hetero-agglutinins in the plasma. He further noted that the cells of some animals (horse, donkey, monkey, dog, rabbit, and goat) were more prone to agglutinate than others (rat, mouse, and guinea-pig). Nattan-Larrier (1910) found the reaction to be accentuated by the addition of sodium chloride and Marty (1917) maintained that a trace of 1 : 1000 potassium carbonate, while it dispersed the pseudo-agglutinated clumps sometimes occurring in other diseases, did not affect the agglutinated clumps in trypanosomiasis. Hornby (1919 b) in studying the suitability of the reaction as a diagnostic test in equine infections found no consistent relation between the presence or absence of the parasite and the existence of auto-agglutination, but concluded that its occurrence to a marked degree suggested the possibility of trypanosome infection. At the present time, most investigators either do not use it as a diagnostic method or consider it of value only as indicating the advisability of further search for parasites.

Autoagglutinins may occur in small quantities in various healthy animals, according to Klein (1902), Landsteiner (1903) and Yorke (1911). They have also been reported sporadically in other infections. Among the parasitic infections, Biglieri (1915) recorded them in serums of 158 out of 600 malarial patients and Marty (1917 and 1917 b) also noticed them in malaria, although to a much less extent than in trypanosomiasis. Nattan-Larrier (1910) found them in canine piroplasmosis to a very feeble degree.

V. *Malaria*

The laboratory diagnosis of malaria rests upon the finding of parasites in blood smears; the chance of this in low-grade infections has been greatly increased by the more general adoption and perfection of the thick-film method which was introduced by Ross in 1903. Nevertheless, many investigators feel that a serological method might be of assistance in detecting infections where the parasites are too scanty to be found in thick films, i.e., in infections of very low grade and during treatment. That antibodies arise during the course of the disease is quite evident from the following review of the literature, but so far no serological test has been perfected to the point where it can be of practical diagnostic aid. As in many other cases, I believe this is almost entirely due to the

difficulty of preparing a suitable test antigen; in this instance the handicap lies in obtaining sufficient malarial organisms comparatively free from host protein. So far, the successful test antigens have been aqueous, rather than alcoholic, extracts of plasmodia-containing material, and their activity has probably been connected with their content of plasmodial proteins.

I. COMPLEMENT FIXATION *

A large amount of the so-called complement fixation in malaria consists, in reality, of various modifications of the Wassermann reaction which are primarily of interest from the standpoint of possible complications in the diagnosis of syphilis. In spite of many claims, however, of positive Wassermann reactions in malaria uncomplicated by syphilis, modern investigators are more and more emphasizing the opinion that with a properly controlled technique, malaria alone does not yield a positive Wassermann. Lloyd and Mitra (1926), in a very illuminating paper on this subject, point out that: (1) the so-called Wassermann test has been carried out in a variety of ways; (2) the highest proportion of positive tests in malaria has been obtained by workers using Wassermann's original method; (3) in many positive tests syphilis has been inadequately excluded, and (4) workers using modern techniques have almost invariably found negative tests in malaria. From their own study of eighty-five cases of malaria these authors concluded that the presence of malarial parasites does not cause a positive Wassermann test provided the technique conforms to modern standards. Readers who are interested in the subject (and other tests for syphilis in malaria) are referred to the bibliography in the paper of Lloyd and Mitra and to the following: Valerio (1911), Bates (1912), de Haan (1913), Zschucke (1913), Fletcher (1914), Gasbarrini (1914 b), Mathis and Heymann (1915), Sutherland and Mitra (1915), Hirsch (1917), de Jong and Martin (1917), Kimura (1917), Meyerstein (1917), Aimé and Lochelongue (1918), Prins (1918), de Jong (1919), J. G. Thomson and Mills (1919), Hehewerth and Kop (1921), Heinemann (1921, 1922), Levy (1921), St. John (1921), van den Branden and van Hoof (1922), Dubois (1922), Chambers (1923), Salvioli (1923), Marcialis (1924), Anorato (1925) and Asbelew (1925).

Among the earlier workers, negative results in specific comple-

* This review is taken largely from papers by the author, L. G. Taliaferro and Fisher (1927) and the author and L. G. Taliaferro (1928).

ment fixation were reported by de Blasi (1907), who used as antigen red blood-cells heavily infected with malarial parasites; by Mircoli (1908), who used extracts of infected red cells; and by Ferrannini (1911), who used both alcoholic and aqueous extracts of infected spleens. Gasbarrini (1913), on the other hand, obtained quite promising results by preparing his test antigen by the earlier method of de Blasi, i.e., heavily parasitized red cells were washed three times in 0.9 per cent sodium chloride, laked in distilled water, dried in a desiccator, pulverized, and later used in a 1:30 dilution in saline. Of the forty-four persons whose serums he worked with, twenty-eight either had malaria at the time or had had a history of it, fifteen had not had malaria (one doubtful) but had other diseases, and one was healthy. The twenty-eight malarial serums when tested were very irregular and chiefly negative, but when they were treated with sheep cells for twenty to thirty minutes, all but four gave positive complement fixation tests. The non-malarial serums, either normal or treated, gave negative results (with the exception of the doubtful case). A control test antigen made from the red cells of a healthy person gave uniformly negative results. Gasbarrini believed that the treatment of the human serum with sheep cells freed the serum of its natural anti-sheep hemolytic antibody by absorption and that this explained the better results with the absorbed serum. In a second paper Gasbarrini (1914) worked with twenty-two cases and concluded that serum from patients during rigor or the febrile stage would fix complement, during latent malaria would fix it weakly and during chronic malaria would not fix it at all.

J. G. Thomson (1918, see also 1919, 1919 c and especially 1919 b) also obtained promising results with an antigen prepared from parasitized red blood-cells by first culturing the material according to his and D. Thomson's modification of Bass and Johns's (1913) technique so as to increase the number of parasites, then hemolyzing the red cells with distilled water, dissolving the sedimented parasites and cell debris in 0.1 *N* NaOH, neutralizing with normal HCl and finally, just before use, diluting with physiological saline until there was no anticomplementary action. He felt that a test antigen from heavily infected spleens in malignant tertian cases might prove even more promising, although the reactions he obtained were rather weak, due to his inability to obtain a heavily infected organ. His preliminary evidence indicated that although there might be a certain amount of specific action, a given test antigen probably showed a group reaction for all species of malaria. Pseudo-positive reactions were obtained with syphilitic serums.

Horowitz-Wlassowa (1924) stated that a test antigen for complement fixation could be prepared from the peripheral blood or placenta of acute malarial infections or from the brain in cerebral localization. To 160 grams of minced placenta was added 100 c.c. of distilled water containing 0.1 gm. of quinine hydrochloride and a few crystals of thymol; the mixture was then shaken, allowed to stand for twenty-four hours and filtered. Following a study of 200 serums from normal and infected persons, this author concluded that a complement-fixing antibody was formed in malaria and was present from two weeks to five years after infection, although during reinfection or relapse, when the parasites and especially the gametocytes were increasing in the blood, it might not be demonstrable; and that the amount of antibody and the space of time necessary for its formation depended upon varying conditions (the duration and intensity of illness, the individuality of the patient, etc.). Of sixty-seven persons giving no history of malaria, the serums of 90.8 per cent were negative. Control test antigens, such as an alcoholic extract of ox heart as used for the Wassermann reaction, Besredka's tuberculosis antigen, echinococcus fluid and an aqueous extract of normal placenta, all gave negative results.

Mirotschnik (1925), using a test antigen prepared from heavily infected blood, obtained the following results with complement fixation on 213 malarial serums: 43 per cent gave strongly positive reactions (+++ to ++++), 30 per cent gave weakly positive reactions (+ to ++)and 27 per cent gave negative reactions.

Savtchenko and Baronoff (1926) used organs from patients infected with malaria and found that the spleen was a mediocre source of test antigen but that alcoholic extracts of the liver proved quite reactive and did not react with syphilitic serums. Unlike other investigators these workers obtained a very great specificity of the complement fixation reaction. Thus, test antigens from a person with *P. falciparum* gametocytes reacted only with the serum from persons showing *P. falciparum* gametocytes and not with serum from persons showing *P. falciparum* rings or *P. vivax*. Similarly, test antigens prepared from the liver of a person showing gametocytes and schizonts of *P. vivax* reacted strongly with the serum of chronic carriers of *P. vivax* showing gametocytes in the blood, and less markedly with the serum of recent cases showing no gametocytes.

Kingsbury (1927) noted that although the complement fixation tests in such disorders as tuberculosis, hydatid disease and schistosomiasis were not so reliable as the Wassermann reaction in syphilis, some success was attained by reducing the amount of comple-

ment to be fixed and by employing a suitable antigen, i.e., one of high sensitivity and specificity and low anti-complementary power. Of eleven antigens tried (eight from spleen, liver, heart, brain and blood of *P. falciparum* infections and three from blood of *P. vivax* infections), the most satisfactory were from saline emulsions of washed and hemolyzed infected blood. The latter gave the following results: *P. falciparum* antigen tested, first, against twenty-five cases of *P. falciparum* gave 48 per cent positive, and later, against twelve cases of tertian gave 50 per cent positive. Two series with *P. vivax* antigen, the first with sixteen *P. falciparum* cases and the second with six *P. vivax* cases, gave 31 and 67 per cent positives, respectively.

In view of the successful results of N. H. Fairley (see page 79) in his complement fixation studies in schistosomiasis with alcoholic extracts of snails containing larval schistosomes, Manson-Bahr (1927) attempted to prepare a test antigen for malaria from the oöcysts of plasmodia in the stomachs of mosquitoes. In spite of the difficulty of obtaining material, he succeeded in making alcoholic extracts of the stomachs of twenty-six infected *Anopheles maculipennis* and twenty-four *Culex* infected with avian malaria, but was unable to find any evidence of complement fixation. He believed, however, that success could be attained with a stronger antigen—at least 100 stomachs to 1.0 c.c. of absolute alcohol. To the present author it seems that he could have secured a stronger antigen had he used an aqueous rather than an alcoholic menstruum for extracting the infected stomachs.

2. PRECIPITIN TEST

The preliminary tests of Ziemann on precipitins in malaria (reviewed in Ziemann, 1924) were unsuccessful. Pewny (1918) obtained precipitates within twenty hours by using as antiserum the serum from patients infected with malaria and an antigen prepared from blood clots (Blutkuchen = placenta?) from a malarial patient. In preparing the antigen the blood clot was mixed with twice its bulk of distilled water, and after digesting at 37° C. for five to six days, was centrifuged, the supernatant liquid pipetted off and diluted with physiological saline. The best dilution was found to be 1 : 100. He obtained negative results when this antigen was tested with fevers other than malaria. Both malarial and non-malarial serums were negative with an antigen prepared in a similar manner from a normal blood clot.

The author has been associated in two rather extensive studies

of precipitins in malaria (see W. H. and L. G. Taliaferro and Fisher, 1927, and W. H. and L. G. Taliaferro, 1928) carried out in Honduras. The first of these involved 1,605 tests with thirty-seven different test antigens on the serums of 535 persons; and the second involved 1,438 tests with seventy-five different test antigens on the serums of 298 persons. We (1927) were unable to prepare reactive test antigens by the method of either Horowitz-Wlassowa or Thomson as used in their complement fixation studies.

In the first year's work our best results were obtained by mincing a placenta heavily infected with *P. falciparum* * in a meat chopper, extracting in an equal volume of ether for several weeks, then extracting the wet ether residue in Coca's solution (an aqueous solution of 0.5 per cent NaCl, 0.05 per cent $NaHCO_3$ and 0.4 per cent carbolic acid) in the proportion of 10 gms. placenta to 50 c.c. of extractive for about a week, filtering through hard filter-paper and using the clear supernatant. The preliminary extraction with ether greatly increased the potency of the antigen, possibly by increasing the permeability of the cell wall, as some botanists have found in obtaining nitrogenous materials from plants (see Chibnall and Schryver, 1921, and Chibnall, 1923), or by removing some lipoidal complex which prevents the solution of parasitic proteins as the author *et al.* have found to be true in schistosome work (see page 83). In the second year's work the same procedure was employed except that the ether-placental material was mixed with an equal quantity of Coca's extractive with the hope of obtaining a stronger test antigen. This resulted in the test antigen becoming acid so that it had to be adjusted to a pH of about 7.8 before use. All of the tests were carried out by the ring test method. Considering only the test antigens just described, eighty-six cases were tested in 1926 and sixty-four cases in 1927. The first series of tests consisted of serums from fifty-four infected persons, which gave forty-five positive (10 +++, 21 ++, 14 +), two doubtful and seven negative tests, and from thirty-two persons, negative for malaria by the thick-film method, which gave six positive (2 ++, 4 +), one doubtful and twenty-five negative tests. The second series contained serums from thirty-two infected persons, which gave thirty positive tests (4 +++, 11 ++, 15 +), one doubtful and one negative, and from thirty-two persons, negative for malaria in thick films, which gave one positive, four doubtful, and twenty-seven negative tests.

* For a description of the localization of *P. falciparum* in the placenta, see Clark (1915).

The fact that the placental test antigens just described were quite perishable led to the attempt to prepare a dried powder from the same source. Whole dried infected placental materials, however, did not yield successful results. Therefore, when a placenta was obtained in which the *P. falciparum* schizonts were in the large segmenting stages, the parasites from it were greatly concentrated by the centrifuging method of Bass and Johns (1915), as can be seen in Figure 3. Working with this dried powder, our most extensive experiments were carried out with a test antigen prepared by digesting 0.05 gm. of the dried powder ten to twenty hours in 1.5 or 2.0 c.c. of 0.05 *N* HCl made up in 0.85 per cent NaCl and using the supernatant after adjustment of its pH (7.6 to 7.8) with 0.05*N* and 0.01 *N* NaOH in 0.85 per cent NaCl. In preparing this type of test antigen, it was essential not to allow the acid to act on the powder over twenty hours, because in our experience a longer treatment resulted in a cloudy antigen and caused a heavy flocculent precipitate in all tubes. Hence, the requisite amount for each day had to be made fresh. With this test antigen thirty-four serums from persons showing parasites in thick blood films gave twenty-nine positive tests (3 +++, 14 ++, 12 +) and five negative, and forty-four serums from persons negative in thick blood films gave nine positive tests (2 ++, and 7 +), three doubtful readings and thirty-two negative. When the dried concentrate was extracted in Coca's solution (0.05 gm. to 2.0 c.c., respectively), twenty-three serums from infected persons gave twenty-two positive tests (12 +++, 9 ++ and 1 +) and one doubtful, and nine serums from uninfected persons gave five positive (1 ++ and 4 +) and four negative tests. In all of our work there seemed to be a group reaction, as *vivax* antiserums reacted just as readily with *falciparum* test antigens as did *falciparum* antiserums.

The chief difficulty of serological work with malaria (as indeed with all serological tests outside of those for syphilis which have reached a high state of perfection) is the standardization of test antigens and technique. In fact, the results of different studies vary so much that no definite conclusions can be drawn. In my own work, for example, having gotten very good results in two studies on precipitins in malaria in Honduras, I felt that it might be possible to standardize the test. In a third series of tests in Porto Rico, however, test antigens prepared, as before, from dried malarial parasites concentrated from infected placentas did not give the promising results obtained in Honduras. To my mind this simply means that a number of unknown conditions which happened to be satisfac-

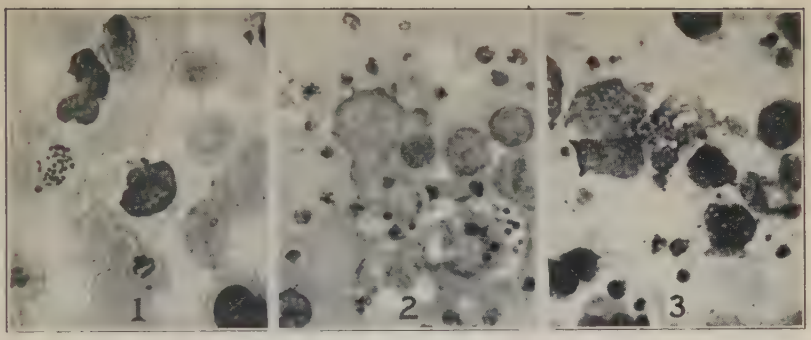


FIG. 3.—Concentration of malaria parasites from placenta: 1, from a blood smear from the placenta; 2, from a blood smear made from the top layer after ten minutes' rapid centrifugation of the minced placenta and blood therein; 3, from a blood smear made from the top layer following a second rapid centrifugation of the residual placental material after the concentrated layer of parasitized cells had been removed. (From the author and L. G. Taliaferro, 1928.)

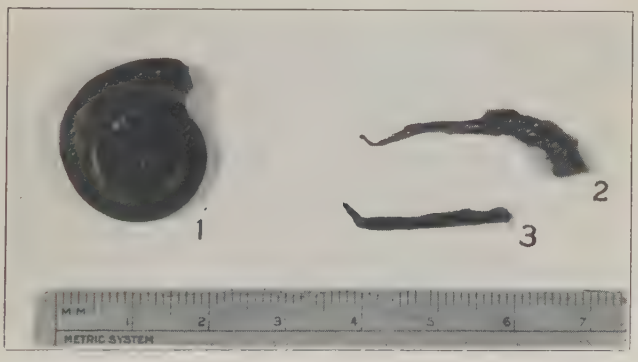


FIG. 4 (see page 82).—A source of test antigens for *Schistosoma mansoni* 1, *Planorbis guadeloupensis*, which is the intermediate host in Porto Rico 2, entire snail after removal of shell; 3, digestive gland or "liver" which contains larval trematodes and from which test antigens are prepared. (From a photograph obtained through the courtesy of Drs. D. H. Cook and W. A. Hoffman.)

tory in the first and second series were not controlled in the third series. Until all such variables are separately worked out, studied and controlled, there is little hope of standardizing a test which will be practical for laboratory diagnosis.

VI. *Coccidiosis*

Serological investigations on coccidiosis have been limited to studies of complement fixation and the non-specific Wassermann test, but have not as yet yielded consistent results.

In regard to the non-specific test, F. Blumenthal (1908) suggested that positive Wassermann tests in "normal" rabbits might be due to coccidiosis. This seemed to be borne out by the work of Kuczynski (1921), who obtained positive results with the majority of infected animals four to eight days after feeding oöcysts, and negative results with normal animals. Marcuse (1922), on the other hand, obtained positive results in only two of thirteen infected rabbits, and very irregular results in twenty-one infected animals when he used the Berlin-Wassermann antigen, and no better results with an alcoholic extract of coccidial nodules of heavily infected livers. Torres (1924) arrived at essentially similar conclusions.

In the specific complement fixation test, in addition to the work of Marcuse just mentioned, Patterson (1923) used as test antigens normal saline, carbol saline and absolute alcohol extracts of infected livers. These were efficient in the order named although the normal saline did not keep well. Tests on the serums of twenty-nine infected rabbits with the saline test antigen gave seventeen strongly positive, nine weakly so, and three negative reactions, while serum of three uninfected rabbits gave negative reactions.

Recently, Chapman (1929) has undertaken a very extensive series of tests in which the most satisfactory test antigen was prepared by mincing and suspending the heavily infected intestine of a rabbit in 50 c.c. of distilled water plus 0.5 per cent phenol, shaking for two hours, leaving at room temperature two days, adjusting to isotonicity with sodium chloride, centrifuging and filtering. From her work she concluded that the positive reactions may have been due to coccidiosis as they appeared too strong to be non-specific and in a few infected rabbits showed an increase in titer, but that the antibodies were of low titer, were slow to make their appearance and were not present in all infected rabbits: thus, the reaction seemed unsuitable for diagnostic purposes.

If a more potent antigen will obviate some of the difficulties, the

recent work of Bachman * may soon yield some interesting results. He has succeeded in isolating coccidia from the heterogeneous mixture of tissue, bacteria and other contaminating elements by applying the technique he devised in his trichina work (page 97). Essentially he found that, by digesting heavily infected intestines in pepsin and hydrochloric acid and then centrifuging, a simple mixture of coccidia and yeasts could be obtained, and that by a differential flotation with a sugar solution, the coccidia could be segregated from the yeasts.

VII. *Schistosomiasis* †

Serological studies in the schistosome infections have progressed to such a point that they will undoubtedly in the near future become very valuable in practical diagnosis.

I. COMPLEMENT FIXATION

Yoshimoto (1910) was probably the first investigator to obtain complement fixation in schistosomiasis. He prepared his test antigen from fresh adult schistosomes from a calf by extracting for twenty-four hours in twenty times their volume of absolute alcohol, centrifuging and before use diluting 1 : 4 with 0.85 per cent saline. Preserved in the ice-box this material kept its potency for two months. He tested fifty serums and one sample of ascitic fluid from forty-three persons with the following very successful results:

	Strong fixation	*Partial fixation*	*Practically negative*	*Negative*
+ Schistosome	10	1	0 1 (ascitic fluid)	0
— Schistosome — Syphilitic			5	14
— Schistosome + Syphilitic	3		5	16

Fujinami and Nakamura (1909) had previously obtained negative results on two infected calves with the same antigen. In one case of

* The author wishes to thank Dr. G. W. Bachman for allowing him to give this progress report of his work before publication.

† This review is taken largely from a paper by the author, Hoffman and Cook (1928).

an infected calf Hayami and Tanaka (1910) obtained positive results with an aqueous extract of the worms. Tanaka (1912-13) tested infected mice, dogs, cattle, horses, and apes with aqueous extracts of male worms and alcoholic extracts of worms of both sexes. Positive complement fixation was obtained with serums from the horse and rat with the aqueous extracts and with serum from the rat with the alcoholic extract. Sueyasu (1916) used both aqueous and alcoholic extracts of adult worms in his complement fixation studies on experimentally infected horses. He obtained positive fixation only in those cases in which the horses had been infected for long periods.

N. H. Fairley (1919 and 1919b: see also N. H. Fairley *et al.*, 1923) did not obtain satisfactory test antigens from adult schistosomes, but devised the ingenious method of obtaining them from the "livers" of snails infected with the larval stages. Furthermore, he discarded saline extracts in favor of alcoholic. His final procedure was as follows: one infected "liver" per 1 c.c. of absolute alcohol (at least 99.1 per cent by volume) was extracted for twenty-four hours at 37° C. during which time it was shaken for three periods of twenty minutes each, and the clear filtrate suitably diluted. Sometimes the alcoholic solution was dried and the residue emulsified by shaking with 0.5 per cent phenol in 0.85 per cent saline. Serums from thirty-two out of thirty-six infected individuals of less than two years' duration and seventy-two out of ninety-seven with chronic infections of over two years' duration gave positive fixation, whereas forty-four syphilitic serums negative for schistosomes gave negative tests.

Murray (1920), working in South Africa and using the local vector as the source of antigen, confirmed Fairley's work. He used 1 gm. of infected "livers" ground with 10 c.c. of alcohol, digested at room temperature for five days with frequent shaking, filtered and mixed with 1 per cent cholesterin (3:2).

Le Bas (1922) came to a different conclusion from Fairley and presented evidence that the antigenic substances obtained from infected "livers" were insoluble in absolute alcohol and acetone, but soluble in saline and 50 per cent alcohol diluted with saline. She believed that the active principle is a protein or associated with protein.

N. H. Fairley (1925), working with dried "livers" *, infected with *S. spindalis* of goats and other ruminants, upheld his former contention that the specific principle is a lipoidal complex and not a protein, since he estimated the actual percentage of alcohol used in

* Two hundred and fourteen "livers" yielded 1.705 gms. or 0.00796 gm. per "liver."

extraction at 96.65 per cent and found that the residue after evaporation failed to give any of the routine color reactions for protein. Furthermore, when he varied the percentage of alcohol, the reactivity of the subsequent test antigen varied proportionally: thus, the test antigen was reactive in dilutions of 1:600 when obtained by extraction with absolute or 90 per cent alcohol, whereas it was reactive only in dilutions of 1:300 when 30 per cent alcohol was used. From further work he concluded that the active principle was acetone insoluble and that extraction of such acetone insoluble material with absolute alcohol removed almost all of the reactive principle, so that subsequent extraction of the absolute alcohol, acetone insoluble residue with either 50 per cent alcohol or saline yielded non-reactive extracts. Moreover, Turner (1926) concluded that Fairley's alcoholic extracts contained no protein, or at most only small amounts, by using Dale and Laidlaw's uterine strip anaphylactic method. Later, Fairley (1927) concluded that these protein-free * cercarian extracts of *S. spindalis* were true antigens, because after daily intravenous injections, three out of four normal goats exhibited a definite, but small, serological response (as tested by complement fixation); a *cured* goat exhibited a similar and slightly higher response, and three infected goats showed an almost immediate and accentuated increase. Moreover, he suggested that such a procedure could be used diagnostically. Thus, if a suspected case be injected with the alcoholic extract and a decided rise in antibody titer take place, this would be pathognomonic of schistosomiasis.

In his paper of 1926, N. H. Fairley devised certain modifications in his preparation of *S. spindalis* antigen. He teased up wet "livers" directly into absolute alcohol (1 c.c. per liver), shook 20 minutes, extracted at 37° C. for twenty-four hours, filtered, concentrated the filtrate in the water bath at 45° C. by bubbling air through the solution until turbid, clarified by the addition of just enough absolute alcohol, stored in 1 c.c. ampoules in the ice-chest until needed, and before use diluted 1:39 with 0.85 per cent saline. In Fairley's experience, a group reaction occurred between the mammalian schistosomes so that *spindalis* antigen could be used for the diagnosis of human infections of *mansoni* and *hæmotobium,* and could also be used to ascertain whether or not a given cercaria was a schistosome; but outside the schistosome group, only *Fasciola* gave a cross reaction. The reaction was studied in goats and monkeys throughout

* In spite of the evidence given, most immunologists will be hesitant in accepting the conclusion that the materials, if antigenic, are actually protein-free.

the course of *S. spindalis* infections by Fairley (1926, 1926 b, 1927 b), and seemed to persist as long as infection lasted.

Tanabe (1923) using an alcoholic test antigen from snails infected with *S. pathlocopticum* obtained positive results with serums from rats infected with this parasite and recorded a group reaction with *S. spindalis*. Cawston (1921) also noted a group reaction between the cercarian test antigens from *S. bovis, S. japonicum,* and *S. hæmatobium*. Höppli (1921) in his complement fixation study of *Fasciola* obtained positive fixation with the serum of one case of *S. mansoni* by using an alcoholic extract of *F. hepatica*. (See also Höppli, 1922.) Bettencourt and Borges (1922) prepared and studied alcoholic antigens from *Fasciola* for use with human schistosomal serum, but found them to be efficient rather as Wassermann antigens, fixing complement in only six out of twenty-three cases of *S. hæmatobium*. Le Bas (1924) obtained essentially the same results.

The recent work of Miyaji and Imai (1928) on complement fixation is reviewed under the precipitin work as it contains some data as to the comparative value of the two tests.

2. PRECIPITIN TEST

Comparatively little has been done toward developing a precipitin test in schistosomiasis. Faust and Meleney (1924) stated that in collaboration with Young and Joffick they have been attempting to develop a precipitin test (similar to the Sachs-Georgi tests for syphilis), but as far as the author knows, these results have not been published.

Probably the first work on a precipitin test is that of Miyaji and Imai (1928), who made a study of both complement fixation and the precipitin reaction in man, experimentally infected dogs and rabbits, and rabbits immunized with worm extracts. The test antigens were (1) finely triturated worms mixed with 0.85 per cent NaCl (1:20), kept at room temperature for two days, centrifuged and the supernatant used as test antigen; and (2) the same except that the powdered worms were extracted with absolute alcohol. In both cases test antigens were prepared from males alone and from mixtures of males and females. The serums of nine rabbits showed various degrees of fixation five, seven, eight, and ten days after immunization (they were all negative previously) when tested with the saline test antigen (which was more efficient than the alcoholic one). They exhibited essentially similar results with the precipitin test. Among nine rabbits and six dogs infected with cercariæ of *S. japonicum,*

in general, the rabbits showed positive responses in from twenty-two to forty-five days after infection and the dogs in from fifty-five to eighty-five days (one dog was tested up to eighty-five days and was uniformly negative). Throughout, the aqueous antigens gave stronger fixation than the alcoholic. Precipitin tests with aqueous antigens were positive on the same day or later than the first positive complement fixation in four of the rabbits, but were definitely positive in only two of the six dogs. In working with human serums, the authors employed principally the aqueous extracts, with the following results:

	Infection: fecal diagnosis	*Complement fixation (65 serums)*			*Precipitin test (46 serums)*		
		+	Partial	—	+	Partial	—
Endemic area	+	16		1	8	2	4
Endemic area	—	15	3	14	6	1	9
Non-endemic area	—			16			16

Twenty-five luetic serums negative for schistosomiasis yielded three positive fixation tests with alcoholic antigens, but were uniformly negative with aqueous ones. From these results the authors concluded that serological tests would uncover cases of schistosomiasis when feces and clinical findings were negative.

The possibility of a precipitin test in *S. mansoni* infections has been recently investigated by the author, Hoffman and Cook (1928). I shall review this work in some detail as it illustrates some difficulties in the preparation of test antigens. In this work we used the "ring" test, as outlined in the introduction to this chapter (page 28), except that the test antigen was used undiluted and in 1:5, 1:25 and 1:125 dilutions. Test antigens were made from dried "livers" of *Planorbis guadeloupensis,* infected with larval stages of *S. mansoni* (see Figure 4). Alcoholic antigens could not be used directly because an appreciable concentration of alcohol coagulates the serum when layered over it, and furthermore, even when diluted, they are such efficient Wassermann test antigens that, although reacting with schistosomal serums, they gave very strong pseudo-positives with syphilitic serums—much stronger than the aqueous extracts which were eventually employed. Aqueous extracts made by directly ex-

tracting the dried "livers" (1 c.c. of extractive to 0.025 gm. powder) with the slightly alkaline solution of Coca, with saline or with 0.4 per cent phenol in saline, yielded specific test antigens. Seventy-nine serums of twenty-eight persons with stools positive for *S. mansoni* gave sixty-three positive (29 +++, 24 ++ and 10 +) and fourteen negative tests; twenty-four serums of five persons known to be negative for both *S. mansoni* and syphilis gave one positive (+) and twenty-three negative tests; seventeen serums of four persons negative for *S. mansoni* but positive for syphilis gave nine positive (2 ++ and 7 +) and eight negative tests.

Although these tests indicated that precipitins were formed we felt that direct extraction of the infected "livers" was not a promising method of preparing test antigens because: (1) We could not standardize the necessary period of extraction. It generally took at least three days but the reactivity was lost after a few weeks. (2) The number of strong reactions was not sufficient. (3) Pseudo-positives with syphilitic serum had to be eliminated. (4) The test antigens were not stable enough and quite frequently showed a spontaneous cloudiness. Accordingly, attempts were made to prepare antigens by extracting the dried "livers" with 0.05*N* or 0.1*N* hydrochloric acid (such as Bachman [1928 b] used so successfully with *Trichina* [see page 97], and we used with malaria [see page 76]) and 0.05*N* or 0.1*N* sodium hydroxide (such as is quite frequently used in bacterial work and such as Thomson used in malaria [see page 72]), and adjusting the supernatant to pH 7.4; but all were unsuccessful. A thorough extraction of the dried "liver" powder with fat solvents and the use of the lipoid-free residue as the source of antigen proved more successful and strongly indicated that the water-soluble material in the dried "livers" is so bound with a lipoidal complex that it is very weakly soluble in water, but when the lipoids are removed, quickly goes into solution. Thus, antigens prepared by a thorough extraction of the dried cercarian powder in a Soxhlet apparatus with ether, absolute alcohol or both, the subsequent extraction of the lipoid-free residue with Coca's solution and the use of the clear supernatant (pH about 7.4 without further adjustment) gave the following advantages over the direct aqueous extraction of dried "livers": (1) Proteins and the reactive material went into solution much more rapidly, so that uniform antigens could be prepared in twenty minutes. (2) The reactions with schistosomal serums were much stronger. Thus, twenty tests on eight known schistosomal serums were all positive (18 +++ and 2 ++), whereas twenty tests on four non-schistosomal and non-syphilitic

serums were all negative. (3) The cloudiness and spontaneous precipitation sometimes encountered with the antigens prepared by direct aqueous extraction were completely eliminated by the preliminary alcohol extraction and largely so by the preliminary ether extraction. (4) Strange to say, the pseudo-positives in syphilitic serums were not eliminated by extractions with alcohol, ether or both. Thus, antigens from the lipoid-free powder gave, in sixteen tests on four serums from syphilitic patients not infected with *S. mansoni,* eight positive tests (3 ++ and 5 +) and eight negatives.

The dried "livers" used in the work just described obviously contained, besides the larval stages, a good deal of snail tissue. It was found, however, that the larval schistosomes could be separated from the macerated liver tissue by centrifugation. This separation was so complete that the serums of two monkeys immunized with the normal snail "liver" gave a +++ reaction with antigens prepared from uninfected snail "livers" and whole infected snail "livers," but a negative reaction with antigen prepared from the concentrated larval schistosomes. Aqueous extracts, prepared from the lipoid-free powder of these concentrated larvæ, acted as potent antigens, and preliminary experiments suggested that they did not give pseudo-positives with syphilitic serums.

Control antigens prepared from lipoid-free powders of normal uninfected "livers" gave uniformly negative reactions with schistosomal serums.

VIII. *Hydatid Disease*

In hydatid disease the clinician is largely dependent upon laboratory findings for a diagnosis. This is strikingly brought out by N. H. Fairley (1922), who stated that even since the introduction of routine X-ray examination, out of 182 patients infected with *Echinococcus,* in an Australian hospital, the case records indicated that the condition had been correctly diagnosed prior to operation in only 40.7 per cent. Hence, more work has been done on this larval infestation than on any other. Three immunological tests have been extensively studied: complement fixation and precipitin and intradermal tests. Of these, most authors agree that the intradermal test, which will be considered in Chapter V, is the best, and of the two serological tests, that complement fixation is the more delicate.

The very extensive literature on the serological tests has been ably reviewed by Weinberg (1913), Zapelloni (1915), Hiles (1926)

and G. Blumenthal (1929), to whom the reader is referred for more extensive details and special papers not mentioned here.

I. COMPLEMENT FIXATION

Complement fixation was first applied to the diagnosis of hydatid disease by Ghedini (1906 and 1907), who used as test antigen the hydatid fluid from human cysts. Following Ghedini's work, the test was used by a series of investigators, most of whom are listed in the foot-note on this page. Among these, especial credit is due Weinberg and his collaborators for their early attempts to standardize the technique. Thus, in 1909, Weinberg came to the conclusion that not only was the method practical for the diagnosis of hydatid but was far more accurate than the precipitin reaction advocated by Fleig and Lisbonne (1908). In 1909 b, he found that persons having had a hydatid cyst retained the specific antibody in their serums long after the cyst was removed; and in 1909 c, he gave a minute description of his technique and results from a large series of tests. As test antigen, he used cyst fluid from either man or sheep, which, after a preliminary testing to preclude the possibility of its fixing complement in the presence of normal serum, was kept in the ice-box or dried. In his actual tests, various dilutions of inactivated suspected serum, guinea-pig complement and antigen in physiological saline were incubated at 37° C. for one hour, the sensitized cells added and the extent of hemolysis noted at the end of another half-hour. In some cases, a rapid diagnosis was made by using fresh serum, thus obviating the inactivation of the serums or the use of guinea-pig complement. He reaffirmed the superiority of the complement fixation test, since normal serums and serums from those with diseases other than hydatid occasionally gave precipitates with hydatid fluid.

In the several reviews of the early work, according to Weinberg (1913), of 306 cases studied, 82.0 per cent were positive, 2.6 per cent slightly positive and 15.3 per cent negative.* According to Ghedini

* The original papers dealing with these cases may be found in Bettencourt (1909, 1910 and 1911), Braunstein (1910), Dobrotin (1910), Eckenstein (1910), Flashman and Butler (1910), de Gaetano (1910), Ghedini (1906, 1907, 1907 b, and 1909), Hahn (1912), Henius (1911), Hertz (1912), Israel (1910), Jianu (1909), Kreuter (1909), Laubry and Parvu (1910), Lejars and Parvu (1909 and 1909 b), Lippmann (1910), Marañón (1910), Match (1911), K. Meyer (1910, 1910 b and 1911), Much (1911), Paisseau and Tixier (1909), Pevnitzky (1910), Puntoni (1910), Putzu (1909 and 1910), Rist, Leon-Kindberg and Parvu (1910), Rosello (1909), Sabrazès, Laffargue and Muratet (1911), Schoo (1910), Skachewski (1911), Strauss (1911), Ströbel (1911), Urioste and

(1913), of over 300 infected persons, 92 per cent were positive; and according to Zapelloni (1915), of 535 cases tested, 88 per cent were positive. From these figures there can be little doubt of the value of complement fixation in hydatid disease, and great reliance has been placed on it. Some authors, however, advise caution in its use. Particularly is this true of some of the investigators who advocate the intradermal tests. In addition, there are others like L. Jaffé (1925) who obtained negative results in four out of five cases, although cysts were later demonstrated.

A general survey of the use of complement fixation in hydatid of animals indicates that the results are not as favorable as with human subjects. This may be due to a real difference in the ease of demonstrating antibodies in the animals tested or to a poorer standardization of the technique. Weinberg and Parvu (1908 b) obtained only three positive reactions from five cases of infected cattle, and similarly, Puntoni (1910) obtained four strong, five weak and eight negative reactions from twenty-one infected cattle. According to Weinberg (1913), he and Parvu obtained positive reactions in all of thirteen infected sheep; but in later work by himself, he failed to obtain such invariable reactions. Positive reactions were obtained in an infected horse and a dromedary by Weinberg and Vieillard (1909) and in four infected pigs by Graetz (1910). Jahn (1920) in tests on the serums from twenty-five infected sheep found eight positives and from thirteen infected cattle only two positives. In hydatid of cattle, Miceli (1922) reported the results as being inconstant, and van der Hoeden (1922) stated that although complement fixation is very useful for the human disease, in animals it is weaker and less often positive.

It has frequently been questioned whether the suppuration and degeneration of a cyst cause the disappearance of antibodies in the serum. On one hand, Ymaz Apphatie and Lorentz (1909), Parvu (1912), Weinberg (1913) and N. H. Fairley (1922) found the reaction was present or even intensified, but Zapelloni (1915) and Pontano (1920) obtained the reverse.

As early as 1911 K. Meyer maintained that cross reactions could be obtained both with serums from persons infected with *Tænia saginata* and hydatid test antigen, and with serums from persons infected with hydatid and *T. saginata* test antigen. Similar results

Scaltritti (1911), Weinberg (1909, 1909 b, 1909 c, 1909 d, 1910, 1911 and 1912), Weinberg and Boidin (1909), Weinberg and Parvu (1908 and 1908 b), Ymaz Apphatie and Lorentz (1908 and 1909), and Zapelloni and Ricciuti (1910).

were obtained by Kreuter (1911), Hahn (1912), Pfeiler (1912) and Bársony and Egan (1912), but they were not obtained by Thomsen and Magnussen (1912) and Sonntag (1913). More recently, the specificity of the test has been stressed. Thus, in N. H. Fairley's (1922) experience, it gave uniformly negative results in

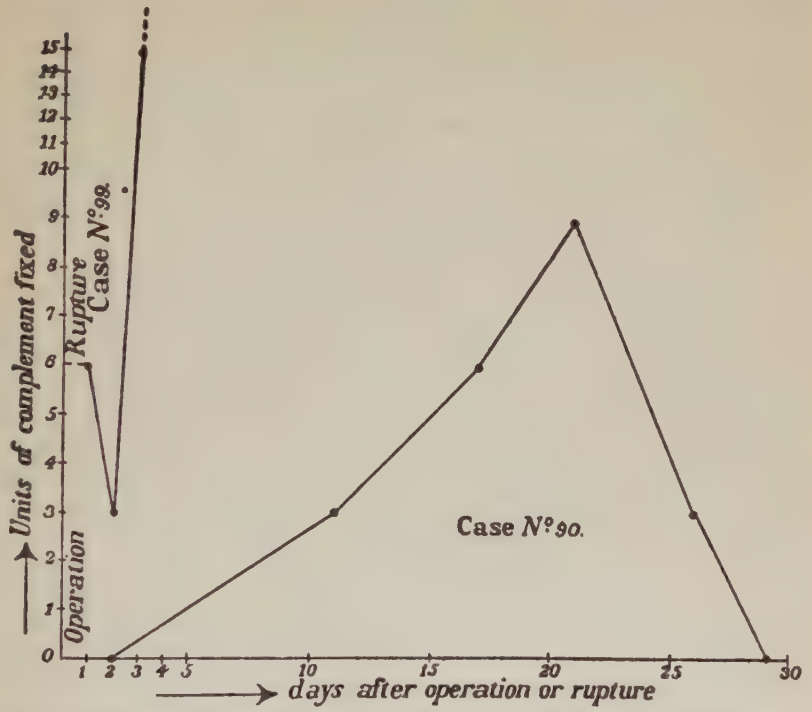


FIG. 5.—Changes in titer of complement-fixing antibody in patients after operation or rupture of hydatid cyst. (From N. H. Fairley, 1923.)

917 patients, some of whom were infected with syphilis or helminths but not with *Echinococcus*, and positive results in seventy out of eighty-three patients infected with hydatid (84.3 per cent). He believed that the non-appearance of the antibody in infected individuals is generally due either to a lack of antigenic properties of the cyst fluid or to a lack of absorption by the host, or, more rarely, to severe intercurrent diseases. This general contention is borne out by the fact that partial or complete rupture of a cyst or operative intervention produces a transient increase in antibody titer. (See Figure 5.) Remé (1927) also reported the case of a patient whose

serum was at first negative for complement fixation, but became positive after puncture of the cyst.

As the development of a standardized efficient test antigen is of paramount importance in any serological test, much work has centered around this point. Numerous procedures have been advocated. The majority of workers have obtained the most successful results by the use of fresh cyst fluid, which some preferably procured from human, others from animal material. There seems, however, to be considerable variation in the reactivity of different fluids (cf. Urioste and Scaltritti, 1911; Duhot, 1919; G. Blumenthal and Unger, 1923; and Thiellé, 1923). Thus, N. H. Fairley (1922) found that four out of eighteen fluids from human operations failed to act as test antigens, whereas over 100 specimens of hydatid fluid from the lung and liver of Australian sheep were efficient.

A constant supply of the fresh fluid is sometimes impracticable. Consequently, experiments with extracts or dried hydatid fluid have assumed a particular importance. Parvu (1909) treated the fluid with five volumes of alcohol and after discarding the precipitate, evaporated the supernatant at 60° C. and resuspended the residue in saline. Weinberg (1913), as well as several other investigators, dried the fluid *in vacuo* and obtained about 1.2 to 1.5 gms. of material per 100 c.c. of fluid. According to N. H. Fairley (1922), Thomsen and Magnussen (1912) prepared an antigen paper, i.e., paper infiltrated with antigen and titrated before use. Some investigators have tried filtration through porcelain filters, but Urioste and Scaltritti (1911) and others found that this lowered its potency. G. Blumenthal (1921) and K. D. Fairley (1923) preserved the cyst fluid with carbolic acid, which seemed fairly efficient. Fairley's results will be described in more detail under the section on precipitins. Some investigators have used aqueous extracts of the cyst wall with more or less promising results (Braunstein, 1910; Heniús, 1911; and M. Rabinowitsch, 1923); and others have tried ether extracts of the cyst fluid (Jianu, 1909; Stenza, 1909; and Falcoriano, 1912).

The most promising results with various extracts have been obtained with alcohol. Kreuter (1911) used an alcoholic extract of the dried cyst fluid, and Chodinski (1916), with a similar extract of the cyst wall, reported complement fixation in half of twenty carriers. N. H. Fairley (1922) found alcoholic extracts active, but never so satisfactory as fresh fluid. With thirty infected cases, Patterson and Williams (1923 and 1924) obtained uniformly positive results with a test antigen made from alcoholic extracts of scolices, subsequently dried and resuspended in saline, in contrast to 83 per cent

positive with fresh fluid; but later Hiles (1926) stated that she understood "that further experience with this form of extract has not quite realized expectations." Bryce, Kellaway and Williams (1924), in a similar comparison of fresh fluid and alcoholic extracts resuspended in saline with serums from 129 infected cases, obtained a reaction with the alcoholic extract alone in seventeen cases, a stronger reaction with it in forty-five, the same results in forty-four, a stronger reaction with the fresh fluid in eight and a reaction with the fluid alone in fifteen. Dew and Williams (1924) reported 80 per cent positives with an alcoholic extract. Hiles (1926) obtained very promising results with a test antigen prepared by extracting dried scolices in absolute alcohol for three days at 37° C. (in the proportion of 1 : 4) which was more efficient than hydatid fluids of animal origin obtainable in England, and retained its potency after twelve months' storage in the dark at room temperature. The active principle was acetone insoluble and was considerably weakened by using saline in conjunction with the alcohol. The test antigen was made more efficient by slowly emulsifying the extract with saline and adding 1 part cholesterol to 1.5-6 parts of extract, just prior to the test. Pseudo-positive reactions with syphilitic serum were eliminated because the concentration of the extract allowed considerable dilution with saline.* Moreover, in agreement with other workers, hydatid serums did not give fixation with Wassermann antigens. Recently, Bryce, Kellaway and Williams (1924) have studied the use of extracts of scolices digested with trypsin.

Since the preparation of an efficient antigen depends largely upon obtaining the active principle † as free as possible from foreign or inert substances, there has been a certain amount of work done with a view to ascertaining its exact source. Although cyst membranes were extracted by Braunstein (1910) and others, the importance of the scolex content was emphasized by N. H. Fairley (1922) and was upheld by van der Hoeden (1923) and Horowitz-Wlassowa (1926), the latter of whom stated that the most active fluid is a cloudy one containing various disintegration products of the scolices, cyst wall, etc. Strongly corroborative evidence was added by Hiles (1926), who found that alcoholic extracts of scolices were efficient test antigens, whereas similar extracts of cyst membranes from which the scolices had been scraped were only feeble.

* Strong alcoholic extracts of cyst membranes can be used as Wassermann test antigens. (See, for example, Pewny, 1921.)

† For a discussion of the chemical nature of the reactive principle, see Flössner (1923) and van der Hoeden (1924 and 1925).

TABLE 13

N. H. FAIRLEY'S COMPLEMENT FIXATION TEST FOR HYDATID DISEASE

Tubes	*1*	*2*	*3*	*4* Serum control*	*5* Complement control*	*6* Antigen control*
Hydatid fluid† (Test antigen)	c.c. 1	c.c. 1	c.c. 1	c.c. 0	c.c. 0	c.c. 2
Patient's serum (1:5) (Antiserum)	1	1	1	1	0	0
Complement ‡	1 (3 M.H.D.)	1.5 (4.5 M.H.D.)	2 (6 M.H.D.)	1 (3 M.H.D.)	1	1
0.85% saline	1	0.5	0	2	3	1
Incubate one hour at 37° C.						
Sensitized sheep cells	1	1	1	1	1	1
Incubate thirty minutes at 37° C.						

* Whenever a series of serums is to be tested at the same time, control tube 4 has to be set up for each serum, but one control tube each of 5 and 6 will suffice for the entire series.

† In addition to the serum to be tested, several known negatives and one known positive serum should be set up to guard against technical errors. Obviously, the negatives should show hemolysis and the positives fixation.

‡ The unit of complement = 3 M.H.D.

The exact protocol used in the complement fixation test for hydatid varies with the investigator. Any of the standard Wassermann techniques, as reviewed by Gilbert (1928), can be suitably modified by using a specific test antigen, etc. In addition, it is interesting to summarize N. H. Fairley's (1922) method with which he has been very successful. As test antigens he advocated (1) clear, undiluted, fresh (no preservative) hydatid fluid obtained from active cysts not showing any signs of degeneration; or, when this was not constantly obtainable, (2) saline or (3) alcoholic extracts of well washed scolices. As antiserum, the patient's serum is diluted 1:5 (1 part serum and 4 parts saline) with 0.85 per cent saline, and inactivated at 55.5° C. for 20 minutes. As complement, fresh serum from a male guinea-pig, after titration in the usual preliminary manner, is diluted so that each unit volume to be used in the main test contains 3 minimum hemolytic doses (M. H. D.). Sheep cells in a 3 per cent suspension, after being washed four times in saline, are

sensitized by adding 4 minimum hemolytic doses of anti-sheep rabbit serum, incubating thirty minutes and afterwards keeping on ice until needed. The final protocol for the test may be found in Table 13. After the second incubation the control tubes 4, 5, and 6 should each show complete hemolysis of the red cells, otherwise the results cannot be considered conclusive. Then, in tubes 1, 2 and 3, no hemolysis indicates a strongly positive serum (fixation of 6 M. H. D. of complement), partial or incomplete hemolysis indicates a weakly or doubtfully positive serum, whereas complete hemolysis indicates a negative. It is to be noted that this method of carrying out the complement fixation test differs from the example given in the introduction to this chapter (page 23), in that in the latter the complement-fixing antibody is titrated by ascertaining what quantity of patient's serum (in the presence of a constant amount of test antigen) is necessary to fix a constant amount of complement, whereas in Fairley's test for hydatid, it is titrated by holding the amounts of both test antigen and patient's serum constant and varying the amount of complement.

In conducting a series of tests it is always necessary to include one known positive and one known negative serum. The known positive can be a human serum that has given strong reactions before. Duhot, Crampon and Lefebvre (1926) suggested as the positive control the use of serum from rabbits immunized with filtered hydatid fluid. This was also used by Alcaraz (1927). In using immune rabbit serum care should be taken to select rabbits which are negative before immunization, as normal rabbit serum sometimes gives positive complement fixation with hydatid fluid. (See Graetz, 1910; and Weinberg, 1913.)

2. PRECIPITIN TEST

Precipitins were probably first successfully demonstrated in the serums of persons suffering from hydatid disease by Fleig and Lisbonne (1907) when they added the serum of a child infected with hydatid to fluid from a human hydatid cyst. Subsequently they made a study of serums from persons both well and affected with other diseases and of rabbit anti-hydatid serum; from this they concluded that the reaction was specific and offered the possibility of being of diagnostic value. Closely thereafter followed the work of Welsh and Chapman (1908) and Welsh, Chapman and Storey (1908). Weinberg (1913) in his tabulation of the results of various authors *

*The original data for this summary may be found in Abadie (1910), Bettencourt (1909), Fleig and Lisbonne (1907, 1908 and 1909), Israel (1910),

TABLE 14

COMPARISON OF RESULTS OF COMPLEMENT FIXATION AND PRECIPITIN REACTIONS IN HYDATID DISEASE (FROM K. D. FAIRLEY, 1923)

Test antigen	*Test*	*Cases*	*Total number*	*Results*				*Percentage of positive reactions*
				+++	++	+	—	
Fresh hydatid fluid	Complement fixation	Proved infection	61	34	3	12	12	81.2
		Probable infection	8	3	2	2	1	
	Precipitin	Proved infection	61	29	8	9	15	75.4
		Probable infection	8	4	2	0	2	
Carbolized hydatid fluid	Complement fixation	Proved infection	62	33	4	8	17	72.9
		Probable infection	8	2	3	1	2	
	Precipitin	Proved infection	62	32	10	10	10	82.9
		Probable infection	8	4	2	0	2	

from 1907 to 1912 showed that of 166 hydatid cases, 30.1 per cent gave positive reactions, 7.2 per cent slight reactions, and 62.0 per cent negative reactions. From an analysis of these data, he concluded that the reaction could not be correlated with the position, size, or number of cysts. These results do not show as good a correspondence as do the complement fixation tests, a fact which is supported by more recent work. Magath (1921) stated, however, that he could find only thirty-seven authentic cases of hydatid tested for precipitins, of which 78.4 per cent were positive, and furthermore, could find no false positives recorded. Indeed, K. D. Fairley's (1923) work, to be reviewed later, indicates that the test can be made as accurate as complement fixation and that its apparent shortcomings may have been due to faulty technique.

Various authors have stressed the specificity of the test. K. D. Fairley (1923) stated that it was absolutely specific. In 270 serums from patients without hydatid he obtained no pseudo-positives except

Marañón (1910), Match (1911), Puntoni (1910), Putzu (1909 and 1910), Weinberg (1909), Welsh and Chapman (1908), Welsh, Chapman and Storey (1908).

for a single plus reaction with three serums in which he could not definitely exclude the possibility of infection.

Filtered hydatid fluid was used almost exclusively as test antigen by the earlier workers, but recently the unfiltered has also been used. Bryce, Kellaway and Williams (1924) believed that the fresh fluid was the best test antigen and that the alcoholic extracts of scolices, dried and resuspended in saline, which they used successfully in complement fixation, were inefficient in the precipitin test. K. D. Fairley (1923), on the other hand, did not obtain such good results with the fresh fluid as with a fluid which he obtained aseptically from the cysts in sheep and to ten volumes of which he added one volume of 5.0 per cent carbolic acid. This possessed several advantages. It assured the preservation of the antigen on ice for as long as six months and prevented contamination in the actual tests. Although this carbolized fluid was superior to the fresh fluid in the precipitin test, the reverse seemed true in complement fixation, a fact which is clearly brought out in Table 14. It is also to be noted from the table that under optimum conditions the two tests are about equally efficient. On the other hand, they do not show a good correspondence with the Casoni intradermal test (delayed type of reaction; see page 185). In consideration of these facts, K. D. Fairley suggested that when the serological test was negative in a suspected case it might be well to perform an intradermal test and *vice versa*.

Just as in complement fixation, the exact technique for the precipitin test varies with each investigator and may be a modification either of a mixing of the reagents or of a "ring" test. Since K. D. Fairley (1923) has obtained such promising results, his method may well serve as an example. Into a small test-tube with a fine bore and a capacity of about 0.8 to 1.0 c.c. is pipetted first about 0.4 c.c. of the carbolized hydatid fluid and then an equal quantity of the serum to be tested, which eventually sinks to the bottom, beneath the hydatid fluid. In the positive tubes, there first appears a haze which gradually becomes definitely a very fine precipitate near the top and later settles to the bottom. The macroscopic precipitate may be present after two hours, but final readings are made after the tubes have stood at room temperature between thirty and thirty-six hours, as follows:

+++ = heavy white flocculent precipitate at bottom of tube;
++ = less definite precipitate with many particles scattered throughout tube;
+ = no precipitate at bottom, but some scattered throughout the tube.

Control tubes in each series consist of: serum and 0.45 per cent carbolic acid in saline; test antigen and saline; and tests with known positive and known negative serums.

In view of the previous work by Dean and Webb (1926) on the importance of the relative proportions of test antigen and antiserum in the precipitin test, Goldsworthy (1928) recorded some very interesting results with the ring test in one case of human hydatid with a test antigen of filtered ox cyst fluid. The first tests, in which the test antigen was layered on undiluted patient's serum, gave practically negative results. On the other hand, a second series of tests, which contained decreasing concentrations of test antigen and various dilutions of patient's serum, showed clearly, as may be seen from an examination of Table 15, that strongly positive reactions could be obtained with the proper proportions of antiserum and test antigen. Thus, the original tests with undiluted antiserum were probably negative because the antigenic content of the hydatid fluid was too low, a condition which could, of course, be obviated by diluting the antiserum or by increasing the antigenic concentration of the hydatid fluid. The author suggested that the unsuitability of certain hydatid

TABLE 15

PRECIPITIN RESULTS* OBTAINED BY VARYING THE DILUTIONS OF ANTISERUM (FROM CASE OF HYDATID) AND ANTIGEN (FILTERED OX CYST FLUID) (REARRANGED FROM GOLDSWORTHY, 1928)

	Dilution of antigen (1 c.c. used)	*Dilution of antiserum (1 c.c. used)*					
		1 in 10		*1 in 50*		*1 in 200*	
		Readings					
		2 hrs.	*19 hrs.*	*2 hrs.*	*8 hrs.*	*4 hrs.*	*7 hrs.*
1	1 in 1	+++	++++	++	Flocculi	+	Medium particles
2	1 in 2	+	+(+)	++	Large particles	+(+)	Fine particles
3	1 in 4	+	+(+)	+	+(+)	++	Fine particles
4	1 in 8	+	+(+)	0	(+)	++	++
5	1 in 16	+	+(+)	0	(+)	+(+)	+(+)
6	1 in 32	+	+(+)	0	(+)	0	(+)
7	1 in 64	+	+(+)	0	(+)	0	(+)
8 C†	1 in 1	0	0	0	0	0	0
9 C	0	+	+(+)	0	(+)	0	(+)

* The number of + signs indicates the degree of opalescence.
† C = Control.

fluids as test antigens, which has been noted by various workers, might be due to similar causes and that the danger of failure in searching for specific antibodies might be minimized by observing the "law of optimal proportions."

3. MEIOSTAGMIN REACTION

The meiostagmin reaction was devised by Ascoli (1910) and was based on the hypothesis that the reaction of an antiserum with its specific antigen lowers the surface tension of the serum. The test "consists of counting the number of drops which flow from the Traube stalagmometer in a unit of time: 9 c.c. of serum or diluted blood is mixed with 1 c.c. of antigen, the antigen being prepared from cyst fluid or cyst membrane. This antigen is an alcoholic extract which may be used either as such or evaporated and taken up in distilled water. The number of drops flowing from the stalagmometer is counted at once, and again after an incubation of two hours at 37° C. Decrease in surface tension, i.e., an increase in the number of drops flowing from the stalagmometer, indicates a positive reaction. The increase is usually about 2.5 to 4 drops [per c.c.]." (Quoted from Magath, 1921.) Working with hydatid disease, Izar (1910) obtained positive results with three pigs and four cows infected with hydatid, and negative results with two pigs and three cows not infected. Similarly, Brugnatelli (1910) obtained positive results with nine infected human serums and negative results with three uninfected controls. In general immunological work, this reaction has not fulfilled the expectations of the original workers. Some of the later investigations indicated that the lowering of surface tension may not be a result of an antibody-antigen union but of a non-specific phenomenon due to changes in the serum as a result of disease. (See Wells, 1925.)

IX. *Trichinosis*

At the present time the diagnosis of trichinosis is made either by finding some stage of *Trichinella spiralis* in the stool, blood or muscle during the various stages of the infection or by indirect means such as clinical symptoms, blood picture, etc. At best, the direct finding of the parasites is complicated by the transient nature of the intestinal and blood stages and the chance distribution of the larvæ in the muscles; and the indirect methods are open to many errors. An immunological test, therefore, with the accuracy of some of those used in hydatid disease, would be of considerable practical importance. The work reviewed in the following paragraphs indicates

that both complement fixation and precipitin tests may be developed for the later or muscle stages and that an intradermal test may be used for the early or intestinal phases of the infection. (See page 186.)

Ströbel (1911), who seems to have been the first worker in this field, prepared his test antigen by mincing trichinous meat, freeing it as far as possible from fascia, digesting it one to two days with pepsin and hydrochloric acid, then centrifuging and washing the sediment several times. This sediment containing many free coiled larvæ and some undigested muscle strands was dried and as such could be kept indefinitely. Alcoholic extracts of this material yielded unpromising results, but sodium hydroxide and antiformin extracts made very successful test antigens for complement fixation tests. The sodium hydroxide extract, which was prepared by digesting 0.2 gm. of the dried powder with 5.0 c.c. of 0.1*N* NaOH for four hours, gave complete complement fixation with the serum of one and strong fixation with the serum of another rabbit, both of which had been infected for three months (a second test with the second rabbit was negative), but no fixation with serums from two rabbits infected for two and seven weeks respectively, and none with serums of one normal rabbit, one normal human and one syphilitic human. The antiformin test antigen, which was prepared by extracting the dried powder with antiformin in conjunction with the alkali for twenty-four hours, neutralizing with HCl and filtering, gave: complete fixation with serums from three trichinous rabbits infected for two and one-half, three, and six months, and from three trichinous humans; strong fixation with serum from a guinea-pig infected for two and one-half months; but no fixation with serums from a normal rabbit, two normal humans, and two syphilitic humans. In the same paper, Ströbel (1911) obtained negative results with the precipitin test, but positive results with the epiphanin reaction of Weichardt. For a discussion of the nature of this reaction, see Wells (1925).

Romanovitch (1912), with a test antigen prepared by grinding muscle heavily infected with trichina larvæ, triturating in a mortar with ground glass and a small amount of saline, placing two hours at 37° C., then over night at ice-box temperature, filtering and using the clear supernatant, obtained negative results with precipitin and complement fixation tests with serums from three rats and three guinea-pigs infected for fifteen days. Ducas (1921) also reported inconclusive results when he used aqueous extracts of infected meat and serums of rats with complement fixation and ophthalmic tests and also with precipitin tests unless large amounts of test antigen were

employed. Of four human cases Strauss (1921) obtained complement fixation in only one, using as test antigen an alcoholic extract of macerated trichinous meat.

Unlike the earlier investigators, Bachman (1928), working in this laboratory, was quite successful in detecting precipitins in rabbits either infected with *Trichinella* or immunized with powders of the parasite. For preparing his test antigen, he used essentially the method of Ströbel, with which, however, he was able to obtain live trichinæ isolated from all host protein; these were dried and powdered; the powder was extracted for twenty-four hours or longer with ether; the ether insoluble portion, obtained by centrifugation, was hydrolyzed twenty-four hours or longer in 0.1 per cent HCl in saline (1 per cent suspension of the powder in the acid) and the supernatant was adjusted to pH 7.2-7.4 before use. Using the ring test, he showed that precipitins were not demonstrable in the rabbits' blood until the thirtieth day, after which they increased in titer to as high as 1 : 3,500 and remained detectable as long as tested (in one case 367 days). One infected human also showed precipitins in his serum with a titer of 1 : 3,500. Furthermore, five rabbits immunized with trichina powder and tested frequently thereafter showed that precipitins were detectable five days after the last injection in high concentrations, remained high (1 : 2,000) until about the fortieth day, but then gradually disappeared until they were not demonstrable after the seventieth day. Corroborative evidence was furnished by tests on eighteen other infected rabbits and seven other immunized rabbits, while neither normal human nor normal rabbit serum nor any of the rabbits before infection or immunization showed precipitins. Bachman's results are particularly noteworthy in that his antiserum reacted with such high dilutions of test antigen. In fact, the titers are higher than the figures show because each original test antigen dilution was expressed as a ratio of the weight of dried trichina powder to the volume of extracting fluid, although only a portion of the powder was soluble. These successful results, in view of the negative results of other investigators, may be due in part to the fact that the test antigens were made from purer trichina material, as Bachman pointed out; but they may also be due to his method of preparation. Thus, his work indicated that the trichina protein is bound with a carbohydrate which is not readily soluble until the carbohydrate is split off by hydrolysis with acid. Ströbel's use of hydroxide as a solvent undoubtedly dissolved the material, but it may have partially altered the proteins, so that they lost a part of their reactivity.

In any case, if these data hold for man, a simple precipitin test

may be devised for detecting established infections, but will probably not be of use in the first stages of the disease (precipitins first appeared only after thirty days) when diagnosis is most needed. Elsewhere, however, is given a review of Bachman's intradermal test in infected rabbits, which becomes positive a few days after infection and which may, therefore, prove effective for the early stages.

X. *Other Helminthic Infections*

In addition to the mass of work done on the particular forms already reviewed, there has been a certain amount of work done on various other species of helminths. Practically, of course, in cases where intestinal worms are now readily and accurately diagnosed by fecal methods, the perfection of serological methods is unnecessary; but, theoretically, the work is of great interest. In general it will be considered, for convenience, in two sections, i.e., the intestinal infections and the somatic infections, unless, as occasionally happens, a paper deals with both types of infection.

I. INTESTINAL INFECTIONS

As early as 1904 Isaac and von den Velden reported a positive precipitin reaction (repeated three times) in a case of *Diphyllobothrium latum* in man, and a negative reaction in an uninfected control, as well as a positive result in a rabbit six days after receiving 20 c.c. of worm autolysate. The test antigen consisted of autolyzed fresh proglottids. In the same year, Fleckseder and von Stejskal (1904) were likewise successful with saline extracts of dried *Tænia saginata* when tested against serums from immunized rabbits. The next year, however, Langer (1905) obtained negative precipitin tests in six *Tænia solium* cases (with homologous extracts), in six *T. saginata* cases (with homologous extracts), in eleven uninfected individuals, ten of whom were sick from other causes, and in infected cats and dogs. In spite of these results, the test antigens, consisting of saline extracts of fresh cleaned proglottids, macerated and preserved in toluol, appeared reactive, since the serum from a rabbit immunized with *T. saginata* gave titers of 1:15,000 with its homologous antigen, 1:6,000 with *T. solium,* 1:4,000 with *Dipylidium caninum,* and 1:2,000 with *Ascaris lumbricoides.*

The first work that I have been able to find on complement fixation is that of Ghedini (1907 b), who found specific complement fixation in the serums of humans with *Ancylostoma duodenale* and *Ascaris*

lumbricoides. This was closely followed by Weinberg and Parvu's (1908) study of forty-one horses in which diagnosis could be made by autopsy findings. Test antigens were prepared from freshly ground parasites or from parasites fixed in absolute alcohol, dried at 37° C. and the powder extracted in physiological saline. *Strongylus,* œstrid larvæ, *Parascaris, Anoplocephala plicata* and *A. perfoliata* were used. Although the authors did not give exact figures, they stated that twenty-one horses showed strong responses for each species of parasite found at autopsy, that some of the others which harbored only a few parasites gave negative or inconclusive results, and that sometimes an animal reacted to test antigens of species other than those found at autopsy, possibly due either to past infection or to a group reaction. They concluded that although the formation of specific antibodies was evidently stimulated, the method of complement fixation would not lend itself to the differential diagnosis of the intestinal worms.

K. Meyer (1910 b) working with carbol-saline and alcoholic extracts of *Tœnia,* obtained complement fixation in persons infected with worms and in rabbits immunized with extracts. There was little species or genus specificity, in that a group reaction could be obtained with *Tœnia, Echinococcus* and *Diphyllobothrium.* He believed the evidence to show that the reactive material in the test antigen was not a protein, but a lecithin-containing lipoid, which was soluble in alcohol, ether and benzol, insoluble in acetone, unaffected by pepsin or trypsin, but destroyed by lipase. A series of further studies dealt with complement fixation, precipitation and anaphylaxis and the nature both of the antigen in tapeworm extracts used for immunization and the nature of the reactive substance in the test antigen. (See Meyer, 1911, 1911 b, 1912, 1913, 1913 b, 1914.) In view of the results reported by N. H. Fairley on a non-protein antigen in schistosomes (page 80), it is interesting that Meyer believed his lipoidal extracts possessed true antigenic properties. He based this conclusion on the evidence that a guinea-pig, injected with an alcoholic extract, although it could not be later anaphylactically intoxicated with a second injection of an alcoholic extract, could be so intoxicated with a saline extract. Similarly, Kellaway (1928 b), working with *Fasciola hepatica* and the uterine strip method, concluded that extracts made with absolute alcohol were able to sensitize guinea-pigs, but were not able to discharge the sensitivity of the sensitive muscle. The theoretical ramifications of these findings are beyond the scope of the present book, but it is interesting to note that Wharton (unpublished work), working in this laboratory, has been unable to repeat

Meyer's results. This fact suggests that Meyer's original alcoholic sensitizing extracts may have contained small amounts of proteins.

Busson (1911) applied complement fixation to a few cases of men and animals infected variously with trematodes, cestodes, both larval and adult, and nematodes. With the exception of a few aqueous extracts, alcoholic extracts of finely powdered parasites were used. Positive complement fixation was obtained in only a small percentage of the cases and negative results were found when the parasites were numerous or had been harbored for a long time. Luetic serums gave positive complement fixation with tapeworm and *Echinococcus* test antigens—a general finding, of course, where alcoholic extracts are used, which has to be ruled out by dilution or parallel tests. Also, normal rabbit serums reacted positively with the various alcoholic extracts, which again is in line with the positive Wassermann findings, as discussed on page 52. According to reviews of his paper, Romanelli (1912) obtained specific precipitins and complement-fixing antibodies in both man and animals infected with various intestinal cestodes and nematodes and in animals immunized with extracts of the parasites. Luigi, Dario and Riccardo (1915) reported positive complement fixation with serums from two *Tænia*-carriers and negative complement fixation with serum from a patient having expelled a tapeworm two months previously and from eleven controls.

TABLE 16

THE CORRESPONDENCE BETWEEN INTESTINAL HELMINTHS IN THE DOG AND COMPLEMENT FIXATION WITH EXTRACTS OF THE SPECIFIC PARASITES (DATA FROM KOLMER, TRIST AND HEIST, 1916)

Test antigen		*Results of complement fixation*					
Species	*Type of extract* *	*Infected with homologous parasites*		*Infected with heterologous parasites*		*Not infected*	
		+	—	+	—	+	—
Tænia serrata	S	3	2	9	58	8	20
	A	4	1	27	40	10	18
Dipylidium caninum	S	2	2	1	67	1	27
	A	2	2	5	63	3	25
Toxocara canis	S	9	27	2	33	5	23
	A	2	34	1	34	3	25
Trichuris vulpis	S	0	34	1	30	1	26

* A = alcoholic; S = saline.

In view of Kolmer's outstanding work in the perfection and standardization of the complement fixation test for syphilis, the paper of Kolmer, Trist and Heist (1916) is of particular interest. They employed nine test antigens consisting of saline and alcoholic extracts of *Tænia serrata, Dipylidium caninum, Toxocara canis,* saline extracts of *Trichuris vulpis, Dioctophyme renale,* and alcoholic extracts of *Tænia saginata,* all of which they tested against the serums from 172 dogs (on 110 of which fecal examinations were made). The saline antigens were prepared by thoroughly macerating 4 gms. of freshly washed parasites, suspending in 100 c.c. sterile saline containing 0.5 per cent phenol, shaking for twenty-four hours, incubating at 37° C. for several days, filtering and storing for use. The alcoholic antigens were similarly prepared except that extraction in the incubator was continued for a longer time. Their data are too extensive to give in detail, but in order to show the correspondence between the serological tests with homologous and heterologous test antigens, and specific infections, I have rearranged part of their data in Table 16. In general, they concluded that antibodies were more likely to be found in infections with the tapeworms, to a less extent with the ascarids, and still less with *Trichuris.* In a study of the specificity of the reaction in five infected dogs, their results indicated a group reaction between the two species of tapeworms, but specificity between the tapeworms and *Toxocara.* Of serums from two persons infected with *Tænia saginata,* one was positive with extracts of this worm and *T. serrata* and to a less extent with *D. caninum,* but negative with extracts of the other parasites used in their study.

A very extensive series of complement fixation tests was carried out by Jerlov (1919) with *Diphyllobothrium* and *Tænia.* Test antigens were prepared in two ways: by the first method, the worms were dried, a 10 per cent emulsion in a mixture of ether and alcohol was shaken for a few hours, the ether was evaporated over a water-bath and alcohol added to make up to the original volume, and the supernatant used; by the second method, the worms were ground with sand and ether, the ether evaporated and saline added to the dried residue so as to make a 10 per cent suspension. Tests were subsequently made using seven test antigens prepared by both these methods from *Diphyllobothrium latum,* its eggs, *Tænia saginata* and a regular Wassermann antigen. In general the results with the specific test antigens seemed to indicate that both types of antigen were reactive in approximately the same degree, that infected persons gave a high percentage of positive tests, and that practically all negative persons gave negative or at best weakly positive tests. Thus, Jerlov

concluded that a positive test definitely indicated infection, but a negative one did not exclude it. Moreover, there was a marked group reaction between *Diphyllobothrium* and *Tænia,* but generally the best reaction was obtained with the homologous extract, whereas carriers of the worms in the absence of syphilis did not give a positive Wassermann test.

Usami (1919), using alcoholic extracts, found positive complement fixation in almost all cases of hookworm infections and group reactions in infections with other nematodes, but consistently negative tests not only in normals, but in persons with tuberculosis and syphilis. Later, Usami and Kamada (1921) reported that perienteric fluid of *Ascaris* gave positive tests with serum from cases with both ascariasis and ancylostomiasis, but that a saline extract of female worms was better.

Violle and Le Saint-Rat (1919) prepared a lipoidal test antigen from *Tænia* by Noguchi's technique. In parallel complement fixation tests with a Wassermann antigen, the two gave exactly similar tests, reacting with luetic serums and failing to react with the serums from patients having *Tænia* and no syphilis and from persons having neither. In view of many similar findings, there can be no objection to the conclusion of the authors that the lipoids of *Tænia* have the same antigenic properties as syphilitic antigens and are, therefore, non-specific. I do not believe that they are necessarily correct, however, in assuming that serums of *Tænia* carriers do not contain specific antibodies, because in preparing their antigen, they may have eliminated or destroyed the reactive substance.

Becker (1920) reported 50 per cent positive complement fixation reactions in fifty-eight persons harboring *Diphyllobothrium latum,* with only 11 per cent positives in ninety-two uninfected persons. Later, the same author (1922) found the Sachs-Georgi flocculation technique to be valueless in testing *Diphyllobothrium*-carriers with test antigens consisting of chlolesterized alcoholic extracts of the worms, since positive reactions were obtained equally well with the serums from uninfected and from infected cases. Le Bas (1924 b) was unable to find either complement-fixing or precipitin antibodies in three persons experimentally infected with *Diphyllobothrium latum,* using saline, alcoholic and acetone extracts of the parasites.

Isbecque (1924) used a test antigen prepared from *Parascaris* of the horse, according to Weinberg and Parvu's method. When tested against the serums of twenty-five patients, the following results were obtained: serums from eight persons harboring *Ascaris* gave seven positive reactions; from three persons infected with both

Ascaris and *Trichuris* gave positives; from six persons infected with *Trichuris* alone gave three positives; and from eight uninfected persons gave only one weak positive. He, therefore, concluded that complement fixation yielded positive results with the serums of persons infected with intestinal worms, and, although not absolutely specific, negative results in uninfected cases.

Recently, Coventry and the author (1928), using as test antigen dried *Ascaris* extracted with the slightly alkaline solution of Coca, found that fifty-three out of ninety serums from natives of Honduras yielded positive tests, but could be correlated neither with *Ascaris* infection nor with other nematode infections. For example, the serums from forty persons infected with *Ascaris* gave 21 +, 4 ? and 15 — reactions, while the serums from fifty uninfected persons gave 32 +, 4 ? and 14 — reactions. The positive reactions in uninfected persons can most plausibly be explained as due to past infection, but in any case, the test can be of little value diagnostically in the tropics where past or present *Ascaris* infection is well-nigh universal. (See Table 27.)

2. SOMATIC INFECTIONS

There have been several studies on complement fixation with *Fasciola hepatica.* Among the earlier workers, Weinberg (1909) and Paccanaro (1909) demonstrated complement-fixing antibodies in the serums of infected sheep. Using an alcoholic extract of the adult flukes as test antigen, Servantie (1921) found that serums from twenty-five sheep infected with *Fasciola hepatica* gave seventeen positive (10 ++++ and 7 +) and eight negative reactions, and serums from eleven uninfected animals gave one positive (++++) and ten negatives. The serum from one human infected with *F. hepatica* was found to give a ++++ reaction, but it also reacted with a hydatid test antigen, although not with a Wassermann one. A positive Wassermann serum reacted with the alcoholic *Fasciola* test antigen. A normal serum reacted with none of the test antigens. Kuwahara and Muto (1921) found complement fixation responses in patients and animals suffering from liver distomiasis to be negative in recent or light infections, but positive in infections of long standing. With an alcoholic test antigen, N. H. Fairley and Williams (1923) found that of the serums of twenty sheep infected with *F. hepatica,* fourteen were positive, two partially positive and only four negative, and that of the serums of twenty supposedly normal sheep, the only one that reacted positively may have been infected because a very detailed examination could not be made. Neither

lung worm infection nor hydatid disease yielded pseudo-positive reactions.

Brocq-Rousseu, Cauchemez and Urbain (1923), however, were unable to obtain a marked correlation between complement fixation and infection with flukes (*Fasciola?*) in sheep. Thus 86-87 per cent of infected sheep gave a positive reaction, but 60 per cent of the uninfected animals also gave it.

Recently, Hoffman and Rivera (1929) studied the relation between precipitins and infections with *Fasciola hepatica* in cattle. Using saline extracts of the insoluble portion of dried and pulverized flukes after treatment with alcohol or ether or a combination of the two, they found that the serums from fifty infected cattle yielded 46 + and 4 — tests and from 50 uninfected cattle (no eggs found in feces) yielded 39 + and 11 — tests. The authors felt the test worthy of more extended work since the few negative reactions obtained with the serums of infected cattle might be explained on the grounds of recent infection and the excessive number of positive reactions obtained with the serums of cattle not found to be infected might, on the other hand, be explained as due to past infection or to a very low-grade infection. Of the various antigens employed, that derived from the insoluble ether residue seemed less satisfactory than the others since it had a tendency to become cloudy during the test.

Reference has been made in the previous section to the work of Busson (1911). Working with *Multiceps scrialis* in the rabbit, Henry and Ciuca (1914) found the pure cyst fluid to be the best test antigen, since aqueous extracts of the macerated adult tapeworm or the membranes and scolices of the cœnurus reacted with normal serums. Tests on ten spontaneously infected rabbits gave no positive precipitin and only two positive complement fixation reactions, but on four experimentally infected animals gave two positive precipitin and four positive complement fixation reactions. These results the authors explained as due to a greater absorption of antigenic material in the experimentally infected animals as compared with the naturally infected ones, because of the larger number of cysts, and because, possibly, of younger infections in which the cysts had not been so completely walled off. In a second paper (1916) dealing with more cases, they found that although there was considerable variability, the antibodies generally appeared in the blood nineteen to twenty-five days after infection. In view of the previous suggestion of Busson it is interesting that they found that the serological reactions might fail in heavily infected cases.

Ando (1917) also found positive complement fixation with

serums from patients with paragonimiasis and dogs infected with the parasite, but not with normal human or canine serums. Incomplete complement fixation was found, however, in cases of phthisis and syphilis.

After studying complement fixation with alcoholic extracts of the moist livers of infected rabbits in man and animals infected with the liver fluke *Clonorchis sinensis,* Ryuji (1922) concluded that the established method of fecal examination was more reliable and easier. Positive tests were obtained with the serums of ten experimentally infected rabbits, three experimentally infected dogs and in 60 per cent of the human cases where the infection was moderate or heavy. Negative results were obtained with the serums of eleven normal rabbits, three normal dogs and eleven humans, some of whom were normal and some infected so lightly as not to show symptoms. No pseudo-positive reactions were noted in cases of syphilis, in infections with *Ascaris,* hookworm or whipworm, or in certain other diseases.

Beckwith and Scott (1924) reported precipitins in about 50 per cent of guinea-pigs, immunized with *Cysticercus tenuicollis* (*Tænia hydatigena*), when tested against sheep and hog hydatid fluid, and partial complement fixation when tested against fresh or saline extracts of scolices.

N. H. Fairley and Liston (1924) have made a very interesting study of complement fixation in twenty-two cases of guinea-worm infection (dracontiasis) in man. Five test antigens were utilized (carbol saline and absolute alcohol extracts of the adult female *Dracunculus medinensis* and of embryos and blister fluid) and the same technique employed as that used by Fairley in his schistosome work. None of the serums reacted with any of the test antigens. Thirteen goats were then injected with varying amounts of extracts of macerated worms and living and dead embryos, but at no time during or after the immunization was positive complement fixation obtained. It would seem from this study, therefore, that neither infection nor immunization with the products of *D. medinensis* stimulates the production of complement-fixing antibodies.

XI. *Insects*

In the myiasis of cattle and horses, Koegel (1924) carried out some precipitin tests (ring test) with *Hypoderma bovis, Gastrophilus equi* and *G. hæmorrhoidalis.* The most efficient test antigen was prepared by triturating five rinsed gadfly larvæ in 100 c.c. of physiological saline and filtering through filter paper. (The antigenic potency

was lowered by boiling the solution or drying the larvæ, and was entirely removed by filtering through a Zsigmondy ultrafilter.) The serums of seventeen cattle before removal of the larvæ gave fifteen positive (2 +++, 10 ++, 3 +) and two negative reactions; and the same after the removal of the larvæ gave fifteen positive (7 +++, 7 ++, 1 +) and two negative reactions; whereas those from sixteen uninfected cattle gave only three positive (+), one doubtful and twelve negative, and from five rabbits all negative reactions. The serums from calves, themselves negative but born of infected mothers, were generally positive, a fact which Koegel explained as due to sensitization *in utero* or to passive transfer by way of the milk. No specificity was detected between the two species of *Gastrophilus*. Cross reactions, but with quantitative differences, were found between *Gastrophilus* and *Hypoderma* and to a less extent with other insects, flies, fly larvæ and lice. No cross reactions were obtained with test antigens from various tape- or roundworms against antiserums of cattle infected with either *Gastrophilus* or *Hypoderma*.

XII. *Summary*

The formation of specific antibodies as a result of infection with protozoan and metazoan parasites has been demonstrated in a wide range of infections.

Omitting the long series of sporadic studies on various infections of man and animals which have not been sufficiently confirmed or are contradictory, the following points are noteworthy: The complement fixation test has reached a high degree of perfection in hydatid disease of man (not animals) and trypanosomiasis (dourine) of horses, and promises to be just as accurate in the schistosome infections of man. It seems probable that simpler techniques, such as the precipitin test, may be perfected for these diseases. Although in no way standardized, as in the preceding, there seems considerable promise that serological tests may be developed in amœbiasis of man and trichiniasis (after the initiation of the muscle stage) of man. Specific antibodies are present in human malaria, but in spite of some very promising reports, the preparation of a standardized and potent test antigen has not been attained. Specific serological tests do not appear to be useful in the diagnosis of kala-azar. Remarkably consistent results, however, have been obtained with several non-specific tests (serum-globulin, aldehyde or "formol gel," and antimony tests) which are apparently

all based on the large increase of euglobulin in kala-azar, with its consequent precipitation in the various tests, and which, with the exception of schistosomiasis, are not positive in a large number of diseases, *including syphilis*.

In general, it seems that the non-specific complement fixation (Wassermann) test for syphilis, with modern methods, is not positive in various parasitic infections of man unless syphilis or yaws is present, but may be positive with the serums from rabbits, either normal, or infected, and to a much greater extent, immunized, with certain trypanosomes.

CHAPTER III*

LYSINS AND REPRODUCTION-INHIBITING ANTIBODIES IN RELATION TO THE TYPES OF INFECTIONS IN TRYPANOSOMIASIS AND MALARIA

I. *Introduction*

In the preceding chapter a description has been given of the work on various antibodies† which, with the exception of the agglutinins, have not been shown to have any functional rôle *per se* in the host's resistance to infection, but which are of paramount importance in the development of serological methods of diagnosis. In addition to these serological responses of the host there are others, such as the lysins and reproduction-inhibiting antibodies, which have a direct effect on the course of the infection, so that in certain protozoan infections their production can be correlated with the type of infection. Such a correlation can be made only in those protozoan infections where the parasites occur in the peripheral blood of the host in all stages and in sufficient numbers to be studied, where subinoculations can be easily carried out and where the host is amenable to such experimental studies. For these reasons work has been limited to certain of the trypanosomes and malarial parasites in laboratory animals, but it is to be hoped that in the future other infections may become available. Even some species of trypanosomes and malarial organisms are unsuitable. For example, *Trypanosoma cruzi,* the causative agent of Chagas' disease, passes a large part of its life cycle in the vertebrate host as an intracellular leishmaniform parasite, and *Plasmodium falciparum* of estivo-autumnal malaria undergoes the major portion of its asexual reproduction within the smaller blood-vessels of the deeper organs.

As a matter of convenience in presenting the present résumé I shall give first the methods which have been developed for analyzing

* The contents of the present chapter are taken, much *verbatim,* from three recent reviews of the subject by the author (1926, 1928 b and 1928 c).

† In thus differentiating the various antibodies, I am simply emphasizing their manifestations.

the effects of the host's resistance * against the parasite, and then, under five selected types of infection, I shall outline, in sequence, the course of the infection, the effect of the host's resistance on the parasites and the immunological bases for the observed effects.

II. *Methods of Analyzing the Effects of Resistance*

Protozoa, such as are considered in this chapter, reproduce asexually either by binary fission or schizogony. The first method is almost universally found in the trypanosomes (Figures 6, 7), although in *Trypanosoma lewisi* at the height of reproductive activity complete division of the cytoplasm may be retarded so that the progeny from a single trypanosome may be temporarily held together as a "rosette" (Figure 7, top section). Schizogony, which is characteristic of the malarial parasites, may be looked upon as a modification of binary fission. Here the nucleus undergoes a series of binary fissions before the parent subdivides ("sporulates"), and consequently a number of progeny are produced at one time (Figure 8).

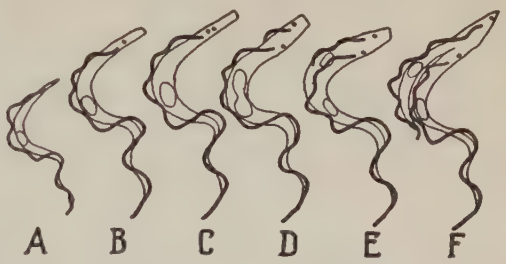


FIG. 6.—*Trypanosoma equiperdum.* Diagrammatic representation of: A and B, short and long individuals resulting from binary division; C, D, and E, stages in growth and division; and F, cell division (x about 1,500). (Modified from the author and L. G. Taliaferro, 1922.)

Granted that a parasite not only has successfully invaded its specific vertebrate host but has started reproduction, it is evident that if no resistance is operative the number of parasites must increase

* As can be seen from the discussions in the present volume, the term "resistance," as applied to the defense of an organism against invading parasites, has many diverse usages and meanings. In the present chapter where a correlation is being developed between the effects of the host's resistance and the types of infection, the term will be used to denote those conditions either active or passive which may arise in the body as a result of infection and which directly affect the parasite. Used in this sense it does not include various possible mechanisms such as defense against the act of invasion, regenerative reactions of the host, or the formation of antitoxic substances. Similarly, it limits discussion to acquired resistance, because as yet experimental data are too meager for a systematic study of the effects of natural resistance.

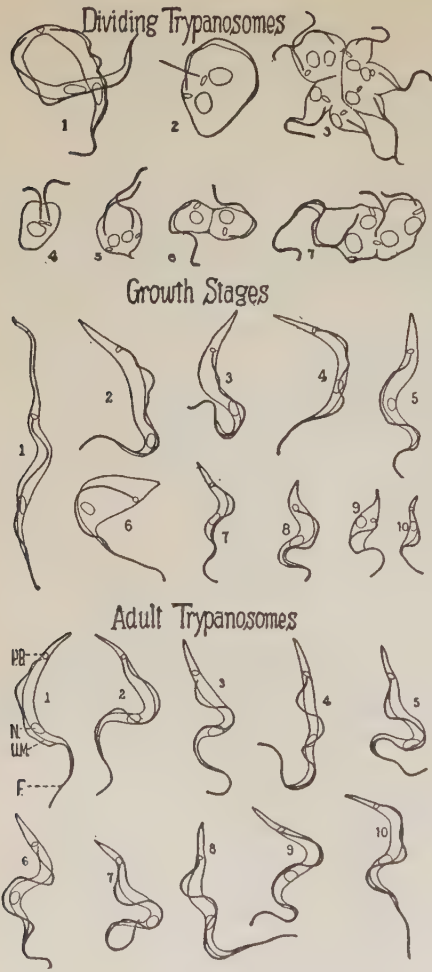


FIG. 7.—*Trypanosoma lewisi. Top:* Dividing trypanosomes such as are found at the height of reproductive activity. *Middle:* Growth stages, taken from the fourth day of the infection shown in Fig. 13. (These are rarely found after the tenth day of the infection.) *Bottom:* "Adult" forms, taken from the twenty-eighth day of the same infection. F, flagellum; N, nucleus; P.B., parabasal body; U.M., undulating membrane (x 1,000). (Top, after Coventry; others, after the author, 1922.)

in the blood at a uniform rate. Also, due to the fission method of protozoan reproduction, such an unmolested increase would cause the number to accumulate according to a geometrical progression series (a, ar, ar^2, ar^3, ar^4, ... ar^n, for example, 1, 2, 4, 8, 16, etc.). If it does not progress uniformly in this manner (and the majority of infections studied do not)—for example, if after the parasite has appeared in the host as an infection, its numbers either remain constant or decrease, it may be concluded that some type of resistance is operative. In other words, the number curves of different infections give an approximate method of ascertaining whether any resistance is operative and an approximate measure of the total effect of this resistance. Parenthetically, it may be noted that the rate of reproduction might *increase,* due to a lowering of the natural resistance of the host, but with little experimental evidence indicating this, it need not be considered here.

Any fluctuations in the number curve, however, can be brought about by one or both of two entirely different mechanisms: (1) the rate of reproduction of the parasites (cell division) may be retarded or inhibited; or (2) the para-

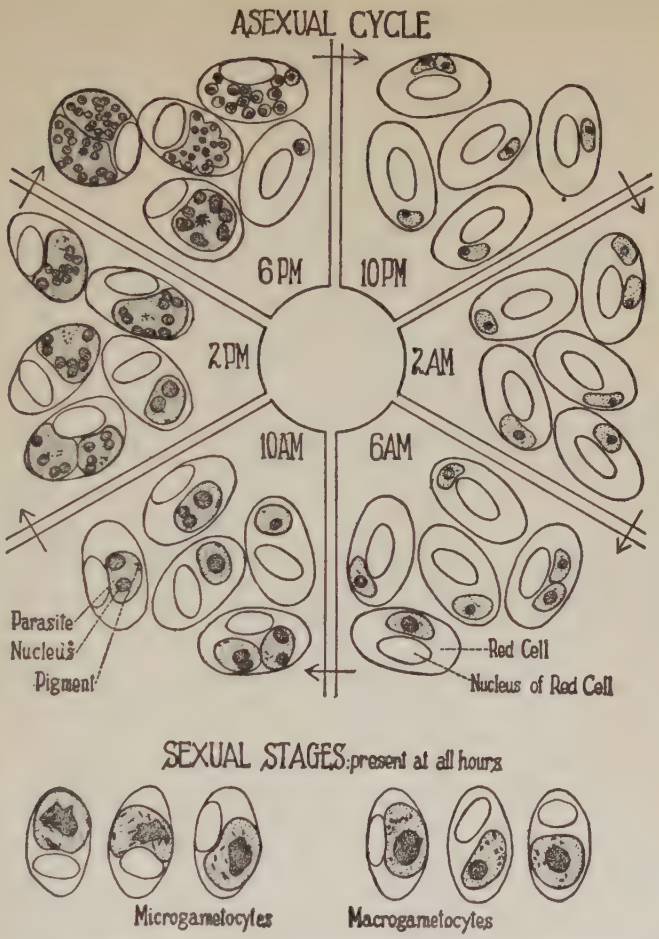


FIG. 8.—Representation of the cycle of reproduction in avian malaria, showing changes in size. Outlines of the asexual stages of the parasites within the nucleated red cells, showing nuclei and pigment granules, made at four-hour intervals during a consecutive period of twenty-four hours. In addition, outlines of three microgametocytes and three macrogametocytes, which occur in small numbers at all hours throughout the infection (× 1500). (From L. G. Taliaferro, 1928)

sites may be destroyed after they are formed. The second mechanism includes those effects of resistance which may be termed *parasiticidal,* but the inhibition of reproduction may not be parasiticidal at all. Practically all discussions of the effects of resistance have hitherto

been limited to the parasiticidal effects. The sharp distinction between these two effects of resistance was probably first clearly brought out by the present author and L. G. Taliaferro (1922), and is indicated in the following equation:

(1)		(2)		(3)
Number of parasites at any time	=	Number produced by reproduction	—	Number destroyed

Here the reproduction-inhibiting effect is represented by the second member of the equation and the parasiticidal effect by the third member. In the work of W. H. and L. G. Taliaferro (1922) and W. H. Taliaferro (1924) on trypanosomes, and L. G. Taliaferro (1925) on avian malaria, the first and second members of the equation have been determined and the third evaluated in the following manner. The first term can be easily obtained by making frequent parasite counts during the course of an infection. The second term of the equation (rate of reproduction), in order to be valid, must be independent of both the first and third terms, and hence, can in no way depend on the enumeration of the progeny formed. Since reproduction in malaria proceeds in cycles, whereas in trypanosome infections it does not, a different measure has been employed in the two groups.

In the trypanosome infections, the measure consists, essentially, in comparing the variability in size of samples of trypanosomes taken at stated intervals throughout the course of an infection. The rationale of this method is based on the obvious and well-known fact that a sample of organisms measured, on the one hand, from a population undergoing rapid reproduction, with the constant production of young forms and intermediate growth stages, will exhibit much greater variability in size than a sample of organisms measured, on the other hand, from a population in which there is little or no reproduction and in which all of the organisms are full-grown adults. (Compare, for example, the variations in length of the reproducing parasites with the constancy in length of the non-reproducing parasites in Figure 9.) Accordingly, from actual drawings of 50 or 100 parasites and measurements of their total lengths, the mean size of samples throughout the course of an infection was determined and from the data the variability was expressed in terms of the coefficient of variation by means of the usual formula. In *T. lewisi*, for example, a coefficient of variation for total length of 3 per cent indicates a population of adults in which there is no reproduction, and a coefficient of variation of 30 per cent indicates a population in

which reproduction is ensuing at a maximum rate. The validity of the method has been discussed in detail in the paper by the author and L. G. Taliaferro (1922). Suffice it to say here that of the various possible difficulties, the most obvious—i.e., that the killing agent is selective against a given age—has been ruled out because there is no change in the mean size just before and just after large numbers

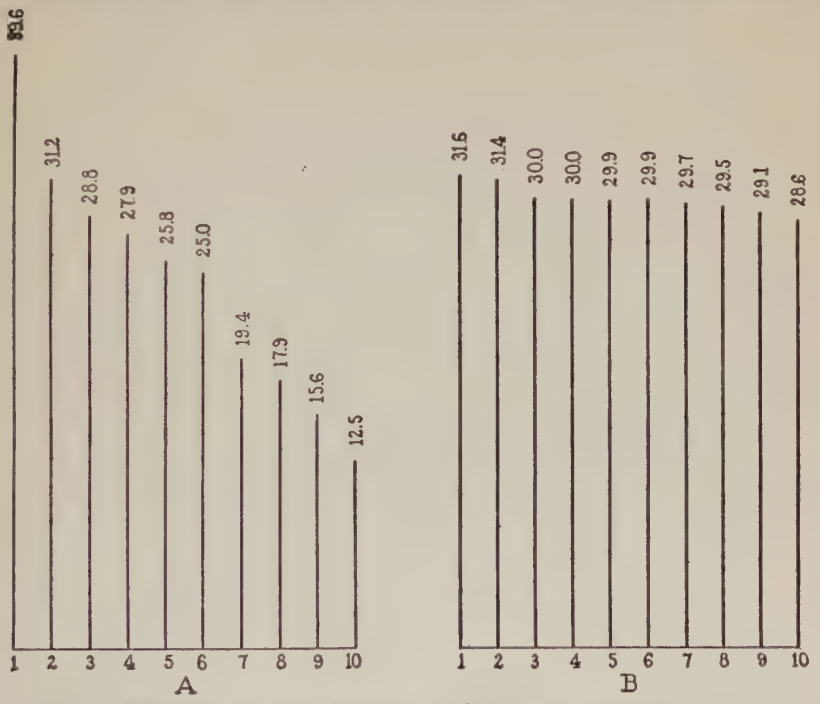


FIG. 9.—Diagram showing the great variability in length of reproducing *T. lewisi* (A) as compared with the constancy in length of non-producing *T. lewisi* (B). These are actual measurements in microns of the "growth stages" and "adult" trypanosomes shown in Fig. 7, and are random samples. (From the author, 1923.)

of organisms have been killed. An approximate method of measuring the rate of reproduction is to ascertain the percentage of dividing forms. This was used by Robertson (1912) and has been a valuable supplementary check in all of our work.

The coefficient of variation method is applicable to trypanosome infections because in these the organisms reproduce by binary fission and do not exhibit any periodicity in reproduction. Thus, if reproduction is going on, a random sample at any time will give all stages

of reproduction and growth. In the malarial parasites, however, the asexual forms sporulate and grow up nearly synchronously (cf. Figure 8). Thus, a sample at one time will contain only small forms (merozoites); at another, large forms (schizonts), etc. The length of time it takes for the organisms to complete this cycle of growth and sporulation is the time it takes for each small organism to grow up and produce about fifteen progeny, and is, therefore, actually an expression of the rate of reproduction of parasites. Should this time vary anywhere during the infection, the rate of reproduction could accordingly be said to vary. It is to be noted that examinations of blood smears to ascertain when sporulation takes place would give a rough measure of the rate of reproduction. A more exact method, which was the one used, is as follows: fifty parasites were drawn, measured and their mean size obtained at two or four hour intervals during as much of the infection as possible. As can be seen from Figure 17, the data obtained show a series of abrupt falls and gradual rises in the mean size of the parasites, due to the fact that when the parasites occurred in the blood as large schizonts, their mean size was high; when sporulation took place, their mean size immediately dropped, but gradually rose, as growth ensued, until the next period of sporulation. By comparing the time it takes this cycle to be completed during the various stages of the infection (acute, chronic, and relapse) it can be ascertained whether the rate of reproduction is changing or remaining constant. In one strain of avian malaria, for example, the cycle was found to take twenty-four hours whenever it was possible to obtain data, and, therefore, the rate of reproduction could be considered as constant throughout the entire infection. This measure of the rate of reproduction presupposes that the *average* number of young produced by each full-grown parasite at each sporulation period does not vary throughout the infection. This has been shown to be a valid assumption by L. G. Taliaferro (1925). Obviously, this method has the same advantage as the coefficient of variation method in the trypanosome infections, in that it is independent of the number of parasites destroyed and can be used as long as enough parasites remain in the blood to obtain a statistically valid sample.

Another method of obtaining the length of the asexual cycle is to ascertain the occurrence of one stage of the parasite at stated intervals. Thus Boyd (1929) used the percentage of sporulating forms in the total population. As sporulation usually occurred between 6 and 8 P.M. in the strain just referred to, this method would give a series of high peaks between 6 and 8 P.M. every day.

III. *Lethal Infections with the Pathogenic Trypanosomes*

1. CONTINUOUS PROGRESSIVE INFECTIONS OF PATHOGENIC TRYPANOSOMES IN THE MOUSE

The simplest type of blood infection is seen when the so-called pathogenic trypanosomes are grown in the mouse. Here, as has been noted by earlier writers on trypanosomiasis (e.g., Massaglia, 1907), the parasites appear in the blood after a short incubation period and increase in numbers steadily and uniformly until the death of the host. Sometimes, but not invariably, the same type of infection is seen in the rat.

This type of infection is illustrated in Figure 10, when *T. rhodesiense*, the causative agent of one type of human sleeping sickness, is grown in the mouse (W. H. and L. G. Taliaferro, 1922). The mouse was injected intraperitoneally with some infected blood. Its blood contained trypanosomes four days later, and from then until the animal died, blood smears were made every twelve hours. The data obtained are shown graphically in the figure. In the first place, the variability of the parasites, as represented by the coefficient of variation, remained fairly high (C. V. = 8.87-10.46 per cent) throughout the infection with no indication of a progressive change. From this it may be concluded that reproduction was occurring at an approximately constant rate. The occurrence of dividing forms in the blood films corroborated these findings. In the second place, the organisms steadily accumulated in the blood and increased according to a geometrical progression. From the constant rates of reproduction and accumulation in the blood it may be concluded that no resistance whatever is acquired by this

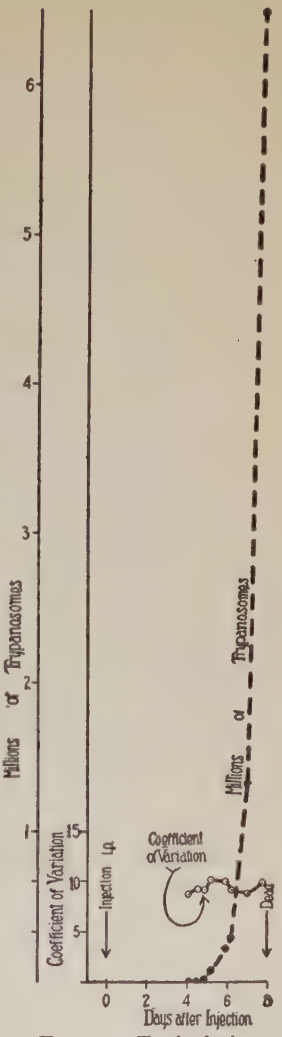


FIG. 10.—*T. rhodesiense* in a mouse. High reproductive activity (see coefficient of variation) and number curve show that neither a reproduction-inhibiting nor a parasiticidal resistance is developed. (From author and L. G. Taliaferro, 1922.)

host, either affecting the rate of reproduction of the parasites or destroying them after they are formed.*

2. INFECTIONS PROCEEDING BY CRISES AND RELAPSES (LYSINS)

When the same pathogenic trypanosomes as were considered in the preceding section are inoculated into guinea-pigs and certain other animals, they exhibit, instead of a continuous and progressive infection, one that is marked by irregular increases and decreases in numbers. The periodic decreases, when the parasites more or less suddenly disappear from the blood, are generally spoken of as "crises," while the subsequent repopulations are termed "relapses." This type of infection has been recognized for many years and is probably very widespread. It is characteristic of *T. rhodesiense, T. gambiense* and *T. brucei* in rabbits, guinea-pigs and rats, according to Fantham and J. G. Thomson (1911) and J. G. Thomson (1912); and of *T. rhodesiense* in man, according to Ross and D. Thomson (1910 and 1911). It has been observed in the dog and frequently occurs when *T. equinum* is grown in rats. An extensive analysis of the infection of *T. evansi* in the rat, using the methods developed by the author

* The statement that this host *acquires* no parasiticidal resistance does not mean that all of the trypanosomes produced by reproduction necessarily survive. In fact, there may be a constant mortality of young forms which is in no sense an acquired resistance, but which may be regarded as the suitability of the normal host as a culture medium. This mortality, therefore, does not affect the present discussion. It does, however, materially affect the validity of the generation time of trypanosomes worked out by Doerr and Berger (1922) and utilized by Kligler and Comaroff (1929). The original investigators noted that infections of *T. brucei* in the mouse increased according to a geometrical progression series. Accordingly, knowing the first and last member of such a progression series and the time between the two, they assumed that the constant multiplier or factor was *two,* due to the method of binary fission, and worked out the time for each generation of trypanosomes. But such a computation is obviously valid only if all of the trypanosomes survive. In this connection the work on avian malaria is particularly pertinent, because in this form reproduction is synchronous, and the generation time can be obtained directly. (It was found to be twenty-four hours in one strain.) L. G. Taliaferro (1925) has been able to show, during the acute rise, when there is an increase according to a geometrical progression series, that whereas the average number of progeny produced at each generation is about fifteen, only about five survive, so that there is a constant mortality of about ten parasites out of every fifteen produced, which represents the suitability of the bird as a culture medium for the parasite. In other words, the constant factor in the geometrical progression series, which *a priori* would have been expected to be fifteen for this form, was actually only five. (For a more detailed discussion, see p. 136 and L. G. Taliaferro, 1925.)

and L. G. Taliaferro, has been given by Knowles and Das Gupta (1928).

The sudden disappearance of the parasites from the blood, coupled with the *in vitro* serological studies to be reviewed later, early convinced investigators that the crises were actually periods of wholesale destruction of the parasites. An examination of the number curve in Figure 11, showing *T. rhodesiense* in the guinea-pig, and a comparison with Figure 10 emphasizes how marked this destruction is. Instead of increasing steadily and rapidly, the parasites were very scarce for twenty-two days and subsequently showed only a few comparatively small rises and falls in numbers until the death of the host on the forty-second day. Therefore, it may be concluded

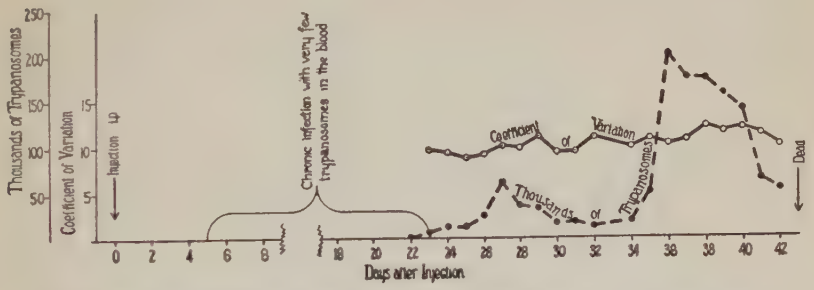


FIG. 11.—Graph showing the course of an infection of *T. rhodesiense* in a guinea-pig. The high reproductive activity (represented by the coefficient of variation curve) indicates that no reproduction-inhibiting resistance is developed, but the type of number curve indicates that a parasiticidal resistance is developed. (From data by the author and L. G. Taliaferro, 1922.)

that a resistance is acquired which destroys the parasites after they are formed. The rate of reproduction, on the other hand, as expressed by the coefficient of variation curve, is strikingly similar in the two figures. Also, daily microscopical examination of the blood films showed cell-division of the parasites to be taking place. Essentially analogous results were obtained in the dog. Therefore, this type of infection may be summed up as follows: Once the parasites are introduced into the host, they probably reproduce at a uniform rate during the entire infection. At intervals, however, most of the parasites that have accumulated in the blood are destroyed, but the few which escape destruction, since their rate of reproduction is unchanged, repopulate the blood again and again until the host dies. Expressed in terms of resistance, in this type of infection which proceeds by crises and relapses, one of the two possible effects of

resistance is operative, i.e., the host's resistance is directed toward a destruction of the parasites after they are formed but not toward an inhibition of the rate of reproduction *per se*.

In all of the author's studies of pathogenic trypanosomes in various laboratory animals, reproduction occurred uniformly throughout the infection. Some of the work of Robertson (1912) on *T. gambiense* in the monkey, however, indicated that an endogenous cycle of reproduction occurs in which phases of active reproduction alternate with phases of lessened reproduction. As was previously noted, she measured the rate of reproduction by ascertaining the percentage of dividing forms on successive days—a method which is undoubtedly valid but which possesses a drawback in that the comparative scarcity of dividing forms makes it difficult to obtain statistically valid samples. To her data, however, which contained the length of trypanosomes from day to day, the author has applied his coefficient of variation method and has found that Robertson's conclusions are sound, but that even at the periods of least reproduction, reproduction is nevertheless occurring at a comparatively high rate. Furthermore, there is some evidence that the original strains of parasites in Africa show these alternate phases of reproductive activity, but that by continuous subinoculation in the laboratory, the rate becomes constant. Consequently, although Robertson's results are of a great theoretical interest, they do not invalidate the general deductions made here, since reproduction was never completely and permanently inhibited, as is the case in *T. lewisi* in the rat.

A long series of investigations are in accord in showing (1) that the wholesale destruction of the trypanosomes at each crisis is due to the sudden acquisition of a trypanolytic property by the host's serum, and (2) that the trypanosomes reaccumulate during the relapse, not because the trypanolysin disappears, but because the trypanosomes become biologically altered so that they are no longer susceptible to the trypanolysin.

The first investigators to recognize the phenomenon of trypanolysis were probably Schilling (1902), Lingard (1904) and Franke (1905). The last also noted that the blood of the host could contain both trypanosomes and strong trypanocidal antibodies, which led him to point out that such parasites had evidently become biologically changed from the original strain and resistant to the antibodies. Credit is due Rodet and Vallet (1906) for the first systematic study of the lysins arising during the course of uninfluenced infections, and their relation to crises and relapses. Massaglia (1907) showed that a definite correlation existed between the

crisis and the lytic property of the serum. Thus, when tested against the original strain of trypanosomes, serum from a guinea-pig before the crisis was only slightly lytic, whereas during and after the crisis it was strongly lytic. Furthermore, serum collected during and after the crisis had no deleterious effect on the trypanosomes reappearing after the crisis. This is clear experimental proof that the trypanolysin which effected the crisis remains in the blood (as shown by the activity of the serum against the original passage strain) and that the trypanosomes accumulate during the relapse because they have become biologically altered, so that they are no longer susceptible to the lytic property. The work of Massagalia was greatly extended by Levaditi and Mutermilch (1909), who showed that trypanolysis, like other immune lytic phenomena, is a "complement-amboceptor" reaction; and by Leger and Ringenbach (1911 and 1912), who found the usual group specificity (Table 35) between a given trypanolytic immune serum and different species of pathogenic trypanosomes. Mutermilch and Salamon (1928 b) have recently returned to a study of the mechanism of the trypanolytic crises.*.

The group reactions found by Leger and Ringenbach in the lysis of trypanosomes by a given lytic serum seem incompatible with the data previously given, demonstrating that a particular lysin will affect only the passage trypanosomes (or the parasites that were present in the animal *before* the formation of the lysin) and not the relapse strains. Thus, on one hand, experimental evidence indicates that the acquired lysins are so specific as to differentiate relapse variants of the same species, and, on the other hand, are so non-specific as not to differentiate between different species. Stated in another way, it indicates that the immunological differences between what are called different species is less than between relapse variants of the same species. This same type of result has been observed in immunization experiments with trypanosomes and is discussed further on page 259.

Simultaneously with the studies of these investigators, others, chiefly Ehrlich and his co-workers, were demonstrating the biological difference between the original strain of trypanosomes and the relapse strain in mice incompletely cured with drugs. Previous to their experiments, Ehrlich and Shiga (1904), Halberstaedter (1905) and Franke (1905) had shown that if a mouse be cured of an infection with a pathogenic trypanosome, it is refractory for about twenty

* For an experimental study of the lysins in human trypanosomiasis, see Heckenroth and Blanchard (1913 b); and in the trypanosomiasis of frogs, see Mendeleeff-Goldberg (1913).

days to a second infection with the same strain of parasites. Ehrlich (1909), Ehrlich, Roehl, and Gulbransen (1909), Rosenthal (1913) and Ritz (1914) found that the original strain of trypanosomes and the relapse strains were so different as to be differentiable by cross immunity tests. Thus, a mouse, infected with the original (passage) strain of trypanosomes and cured, is refractory to a second infection with the passage strain, but can be infected with a relapse strain and vice versa. With the same methods, Ritz (1916) differentiated the original and relapse strains arising during the uninfluenced course of an infection in the rabbit.

Of the papers mentioned, the one of Ritz (1914) is of particular interest. One of his mice was incompletely cured twenty times with the production of seventeen immunologically different relapse strains. Some of these strains were identical with those of another mouse which had been incompletely cured nineteen times with the production of nine immunologically different strains. Thus, when the relapse strains in each mouse were numbered consecutively he found that No. 7 of the first mouse was the same as No. 3 of the second, and Nos. 6, 9 and 8 of the first were the same as Nos. 4, 5 and 11 of the second, respectively. In over 600 mice he found twenty-two immunological variations.

In the work that has just been reviewed the original infecting strain and the various relapse strains have been differentiated by cross immunity reactions and by their resistance to lysins. Rieckenberg's blood platelet test seems to be another valuable method of differentiating the original from the relapse strain. Originally, he (1917) found that when the citrated blood of a rat cured of nagana (*T. brucei*) is mixed with blood containing homologous trypanosomes, the trypanosomes are loaded with blood platelets within a few minutes to such an extent that their movement is impeded. The actual test is carried out by placing a drop of blood from the cured animal on a slide, mixing with one drop of citrated bouillon (faintly alkaline nutrient bouillon + 2 per cent sodium citrate) and adding the trypanosomes to be tested. On microscopic examination in a positive reaction the trypanosomes are agglutinated and the blood platelets adhere to them, generally in fifteen to twenty minutes. It is absolutely necessary that no clotting of the blood from the immune animals should take place. When serum taken from clotted immune blood is used in conjunction with platelets either from an immune or normal animal, agglutination of the trypanosomes occurs, but not the blood platelet phenomenon. Rieckenburg considered, but gave evidence against, the view that the test is a phase of the

well-known agglutination phenomenon. Furthermore, his experimental data indicate that the test is dependent upon a specific peculiarity of the blood platelets of the immune blood. He found that the reaction occurred in rats, guinea-pigs and rabbits which were cured or chronically infected and that it was so specific that it was observed only with the homologous strain. (He used five different strains.) The reaction served to differentiate passage and relapse strains and was present in all cases where immunity against reinfection existed. This work has been extended by Brussin and Beletsky (1925) and the criterion used in a study of the immunity to superinfection in trypanosomiasis (page 252). Rieckenberg originally supposed that this phenomenon was not due to an antibody, but was evidence that one had been formed. Leupold (1928), however, has been able to transfer the property passively to a normal mouse by means of the serum taken from an infected mouse.

It is essential in all of this work, wherever strains are to be differentiated, to keep the original passage strain of trypanosomes (and any subsequent strain to be tested) continuously in such an animal as the mouse, where no crises occur and where the parasites do not come in contact with lytic antibodies and hence do not become biologically changed as they do after each trypanolytic crisis in such hosts as the guinea-pig, dog, rabbit, etc. Failure to do this probably explains the peculiar results of Kligler and Weitzman (1924), who failed to find any evidence of the formation of humoral antibodies in experimental trypanosomiasis. They have, however, added the interesting experimental finding that the injection of olive-oil brings about relapses. This work will be reviewed in connection with the cellular phases of immunity (page 240).

The acquisition and inheritance of antibody resistance by the trypanosomes is a biological problem of great interest. Similar resistances to antibodies and lethal drugs have been observed in many parasitic and free-living protozoa. (For a general review of these "induced variations" in the trypanosomes, see Dobell, 1912; and for similar work on a free-living species, *Paramecium*, see Jollos, 1921.) A remarkable feature of the resistance is that it may be inherited for many asexual generations, although in time it is lost. Furthermore, a radical change in environment, such as passage through the invertebrate host or through another species of vertebrate host, is followed by loss of the resistance. Trypanosomes, to become resistant, do not have to be in the body, as a few minutes' contact between the organisms and the immune serum produces a resistant strain (Ehrlich, Roehl and Gulbransen, 1909, Levaditi and

Mutermilch, 1909, and Levaditi and McIntosh, 1910). Among the explanations offered for the mechanism of this acquisition, the following may be noted: Ehrlich's explanation formed an integral part of his side-chain theory of immunity and is too well known to need discussion. In brief, he held that the nutriceptors of the original strain combine with the trypanocidal antibodies and atrophy, whereupon new receptors are developed by the parasites, which will not combine with the antibodies and hence leave the new parasites resistant. (Ehrlich's explanation of the formation of chemo-resistant strains differed from this explanation in that, instead of an atrophy of the nutriceptors, he postulated a modification of the corresponding receptors.) Levaditi and Mutermilch (1909 b), Levaditi and McIntosh (1910) and Mutermilch and Salamon (1928) believed that the phenomenon was a process of selection in which the non-resistant strains were killed by the antibody, leaving the resistant ones. If they mean by this the selection of certain genetical strains from a "wild population" of mixed strains, their hypothesis is not tenable, as the phenomenon is produced within a single cell strain, the individuals of which are presumably of the same genetical constitution. Nevertheless, at each crisis when the resistance is acquired, there is obviously a selection, but one selection is effective—a fact in marked contrast to the slow effects of selection observed by various authors working with the free-living protozoa. (See Jennings, 1920.) Finally, Rosenthal (1913) believed that there was a substance in the serum which had the specific property of producing relapse strains and which could be differentiated from the lytic antibody by (1) the lack of parallelism between the titer of the two, (2) differences in thermolability, and (3) differences in the fractions of serum in which they are precipitated. It is significant to note that in the case of the formation of chemical-resistant strains from single-cell strains of *Paramecium,* according to Neuschlosz (1919-20), the acquisition of a resistance to a chemical is associated with the acquisition of the power to destroy the specific chemical or to convert it into a less poisonous compound. Jollos has classified all of these cases of acquired resistance, together with certain other variations which are inherited for a greater or less time, as persistent variations or "Dauermodifikationen" and has thus differentiated them from true mutations.

It would seem probable from the work just presented that if lytic serum were injected into mice infected with the passage strain of organisms, an artificial crisis should follow similar to that seen in the natural infection in the guinea-pig. Strange to say, most

earlier investigators failed to obtain such results. Thus, with the injection of 1.0-2.0 c.c. of serum from guinea-pigs during a crisis, Massaglia (1907) failed to increase the length of life of mice infected with *T. evansi* over that of a control. Diesing (1905), however, reported the temporary disappearance of parasites and clinical improvement of cattle and horses receiving serum from asses after recovery. Kleine and Möllers (1906) produced artificial crises in mice infected with *T. togolense* (= *T. brucei*). Recently, the author

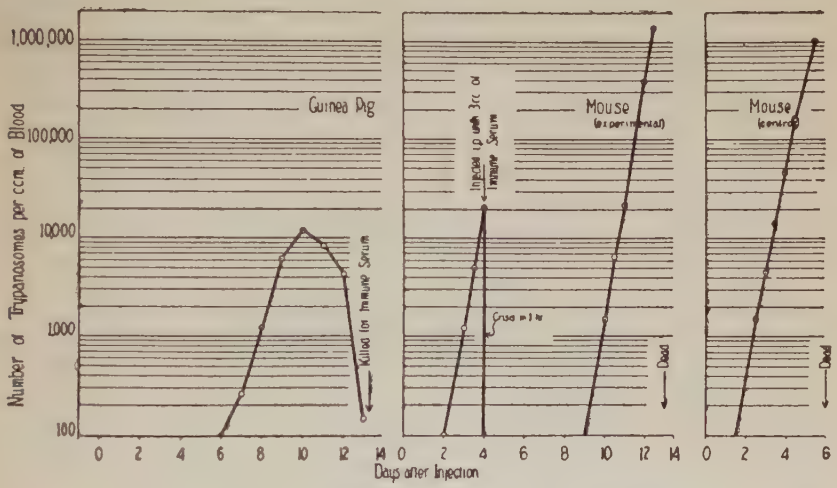


FIG. 12.—Production in a mouse, infected with *T. equinum*, of an artificial crisis as the result of the injection of serum taken from a similarly infected guinea-pig after a natural crisis. The first graph shows the course of the infection in the guinea-pig and the time of bleeding for immune serum. The second graph shows the course of the infection, with a crisis and subsequent relapse, in a mouse given 0.3 c.c. of the guinea-pig serum. The third graph shows the course of the infection in the untreated control mouse. (From the author and T. L. Johnson, 1926.)

and T. L. Johnson (1926) working with *T. equinum* produced artificial crises in mice infected with the passage strain by injecting lytic serum, obtained from guinea-pigs and rabbits after a trypanolytic crisis, from a sheep during its chronic infection, and from mice after incomplete cure with drugs. Figure 12 shows an experiment in which lytic guinea-pig serum was used. Following infection with the passage strain and a crisis, the guinea-pig (first part of figure) was bled and its serum preserved on ice until ready for use. In the meantime, two mice had been infected with the same

strain, and when the parasites appeared in the blood, one (second part of figure) was injected with 0.3 c.c. of the guinea-pig serum, while the other (third part of figure) was left untreated as a control. Within an hour, no trypanosomes could be found in the experimental mouse, nor did they reappear for six days. On the seventh day they were found in small numbers, and thereafter increased steadily until the animal died. In marked contrast, the control mouse showed an uninterrupted increase of parasites until its death on the sixth day. Thus, an injection of 0.3 c.c. of immune (lytic) serum not only produced a six-day crisis but prolonged the mouse's life seven days over that of the control. This work will be considered in more detail under the curative action of immune serums (page 156). At that time a discussion will be given of the possible reasons for the failure of the earlier investigators.

IV. *Infections with the Non-pathogenic Trypanosomes (Lytic and Reproduction-inhibiting Antibodies)*

Trypanosoma lewisi is a representative of a large group of trypanosomes, occurring in various species of rodents, which are very similar in structure and are non-pathogenic to their hosts. Careful enumerative studies of the form by Steffan (1921), fully confirmed by the author and L. G. Taliaferro (1922), the author (1924) and Coventry (1925), indicate that the course of infection in the blood proceeds as follows (Figure 13): After an incubation period during which no parasites are found, the organisms increase rapidly in numbers, sometimes attaining several hundred thousand per cubic millimeter of blood; then there is a crisis when most of the parasites are destroyed; those that remain continue to live in the blood (from several weeks to several months) until a second crisis sweeps them too from the blood. Rabinowitsch and Kempner (1899) showed that thereafter the rat is immune to a second infection—an observation which has been repeatedly confirmed by other investigators.

Many years before exact enumerative studies were made on infections with *T. lewisi*, it was noted from microscopical studies by L. Rabinowitsch and Kempner (1899), v. Wasielewski and Senn (1900) and Laveran and Mesnil (1901) that when the organisms were increasing in the blood they were actively reproducing by fission, whereas during the latter part of the infection they simply existed in the blood as non-reproducing adults. These facts were studied more intensively by the writer with the assistance of L. G. Taliaferro

(1922). From daily blood smears throughout the course of an infection with *T. lewisi* the total lengths of a hundred parasites were drawn and measured and the coefficients of variation computed, as outlined previouly, to obtain an index of the rate of reproduction. From these data, it was found that when the organisms first appeared in the blood they were reproducing at a very rapid rate, but that their reproduction was quickly but progressively retarded until finally, about the tenth day of the infection, no reproduction was taking place at all, and all of the parasites were in the adult stage (Figure 7 and the coefficient of variation curve in Figure 13).

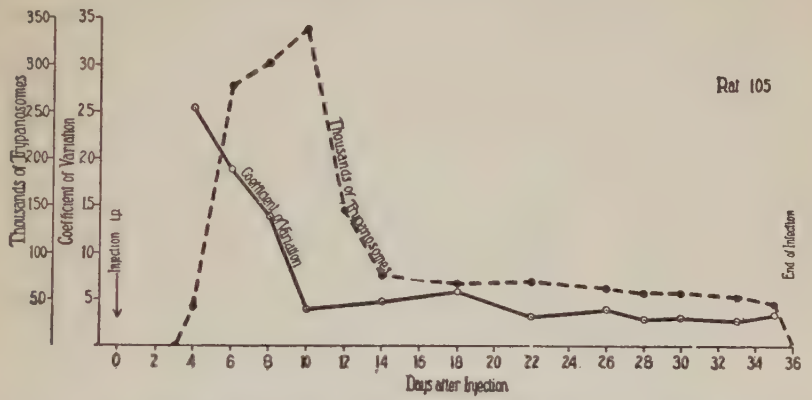


FIG. 13.—Graph showing the course of an infection of *T. lewisi* in a rat. The changes in reproductive activity (represented by the coefficient of variation curve) and the type of number curve show that both a reproduction-inhibiting and a parasiticidal resistance are developed. (From the author and L. G. Taliaferro, 1922.)

From this work it may be said that there are three manifestations of resistance: (1) the retardation and final inhibition of reproduction by about the tenth day, (2) the sudden destruction of the majority of the parasites between the eighth and twelfth day, and (3) the eventual total destruction of the parasites which terminates the infection in from a week to several months.

The immunological basis of the first effect of resistance has been studied by the author (1924) and seems to be due to the acquisition of an immune property by the serum of infected rats which inhibits cell division but which does not kill the parasites. The method of demonstrating the presence of this property is shown in Figure 14, which is typical of numerous experiments carried out by the author

and confirmed by Coventry (1925). On the tenth day after infection (when the trypanosomes had reached the adult stage) a rat (seed rat 972, in figure) was killed and bled and its serum containing the

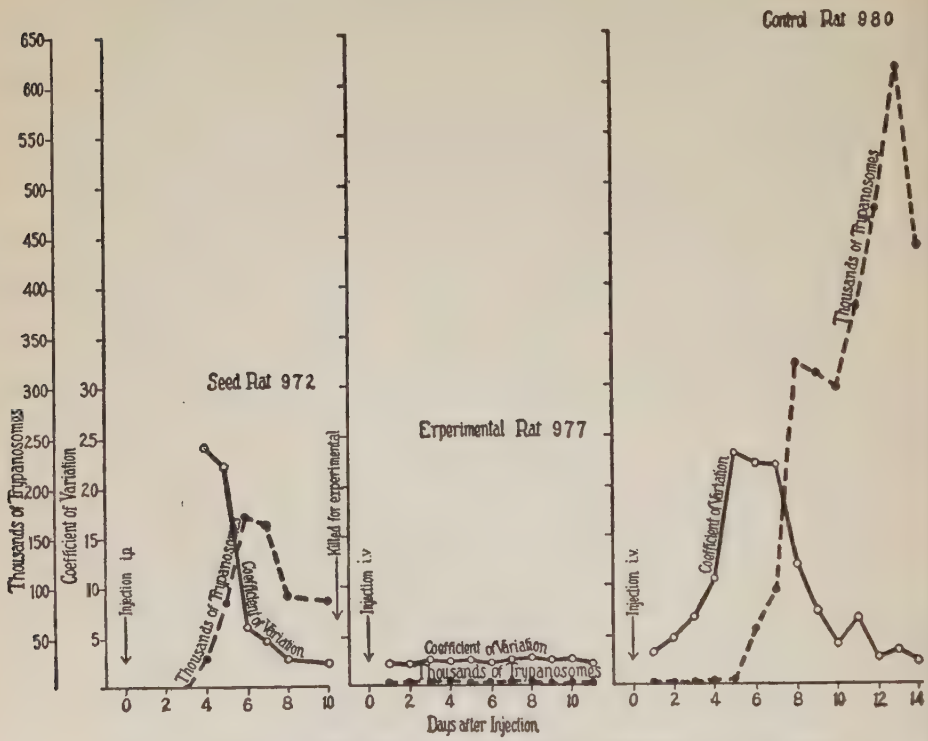


FIG. 14.—Graphs demonstrating the passive immunization of an uninfected rat with the reproduction-inhibiting reaction product which has been formed in an infected rat. Note that after reproduction had been inhibited in seed rat 972, one-half of its adult trypanosomes, plus a suitable amount of its serum, was injected into experimental rat 977, while the other half of its trypanosomes, plus an equal amount of serum from a normal rat, was injected into control rat 980. Subsequently, in experimental rat 977 the normal cycle of reproduction was altered—there was a complete inhibition of reproduction as evidenced by the coefficient of variation; whereas in control rat 980 the normal cycle of reproduction took place. (The peak in the number curve of control rat 980 is so much higher than that of seed rat 972 because it received an enormously larger number of parasites initially.) (From the author, 1924.)

adult trypanosomes collected. Next, the trypanosomes were separated from the serum by rapid centrifugation. Then half of the adult trypanosomes together with 2 c.c. of the serum (per 100-gm. rat) were injected into experimental rat 977, while the other half of the

trypanosomes together with a similar dose of normal rat serum were injected into control rat 980. Daily examinations of blood smears and calculations for the coefficient of variation for total length for each of these rats showed that the trypanosomes in experimental rat 977 lived in the blood for eleven days (end of infection) without showing any reproduction whatever, whereas in control rat 980 reproduction began on the second day and followed the course of a normal infection. In this experiment it is to be noted that not only did the immune serum (i.e., the serum taken from the seed rat on the tenth day) prevent adult trypanosomes from reproducing in a fresh rat, but that normal rat serum failed to prevent adult trypanosomes from reproducing in a control rat. This is a clear-cut case of the passive transfer of this type of immunity from an infected to an uninfected rat.

The changes in titer of this immune property of serum through the course of an ordinary infection have been studied in detail by Coventry (1925). She was unable to demonstrate its presence in serum prior to the fifth day of the infection although it was probably present, as the rate of reproduction had already begun to decline; but between the fifth and sixth days she found a sudden and very great increase with a subsequent gradual increase until the thirty-fifth day, and then a gradual decrease.

As this immune property of serum which inhibits reproduction without affecting the viability of the parasites emphasizes a new effect of resistance, the author has attempted to compare it with the well established immune bodies (1925 and unpublished work). Like most antibodies, it is specific and has no effect on the pathogenic trypanosomes, is non-ether soluble and is precipitated in the globulin fraction of the immune serum (with both the euglobulin and pseudoglobulin). It differs from them, however, in its lack of *in vitro* affinity for its supposed antigen. Thus, when serum containing it is left for twelve hours in contact with large numbers of dividing *T. lewisi* and the parasites are subsequently removed, the serum does not lose any of its titer. Corroborative evidence to the same effect is furnished by the fact that in the usual technique of transferring an infection from rat to rat, which involves more or less contact between parasite and immune serum *in vivo*, the parasites are not sensitized, but proceed to establish a new infection with the consequent reproducing forms. Because of this fact, according to some investigators, this reaction product should be classified as a "reagin" instead of an antibody; such a viewpoint, however, is largely a matter of definition.

Regendanz and Kikuth (1927) have verified the author's conclusions in regard to the formation of the reproduction-inhibiting reaction product in infections with *T. lewisi,* and similarly, found that it exerted no trypanolytic or trypanocidal activity. They have added the important finding that splenectomy has a profound effect on its formation (see page 236).

Recently, Coventry (unpublished work) has found that the second manifestation of resistance, i.e., the first number crisis which occurs between the eighth and twelfth day, is due to a transferable parasiticidal substance, since serum from rats after this crisis while they still contained trypanosomes was trypanolytic in *in vivo* curative experiments. Regendanz and Kikuth (1927) have not encountered this number crisis, but we have invariably found it in the hundreds of infections studied in this laboratory.

Some work on the immunological basis for the immunity of recovered rats to a second infection indirectly indicates that the third manifestation of resistance, i.e., the crisis which terminates the infection, is due to a phagocytosis or a lysin or both. Laveran and Mesnil (1901) considered the final disappearance of the trypanosomes as due to a phagocytosis of the living parasites; but MacNeal (1904), Manteufel (1909) and the author (1924) failed to demonstrate phagocytosis and concluded that a trypanolysin was formed. Recently, Regendanz and Kikuth (1927) maintained that after the reproduction of the parasites was inhibited, there was no sudden disappearance of the parasites, but that various non-specific agencies, such as phagocytosis by the reticulo-endothelial system, continuously removed the parasites. Coventry (unpublished work) found recently that the sudden removal of the parasites from the blood-stream is associated with a transferable parasiticidal substance, probably a lysin. It is not at all improbable that there is no conflict between the findings of Laveran and Mesnil and those who believe a lysin is responsible, since both may be dealing with the same antibody. (For a full discussion of the relation between phagocytosis and lysis, see page 132.)

A number of authors have shown that *T. lewisi* will infect guinea-pigs although the infection does not last so long nor are the parasites so numerous as when grown in their natural host, the rat. Coventry (1929 b) has found that the general course of the infection is essentially similar to that of *T. lewisi* in the rat as given in Figure 13. Furthermore, in hyperimmunized guinea-pigs she has been able to demonstrate the reproduction-inhibiting antibody and has passively transferred it to rats.

V. *Comparison of the Lethal and Non-lethal Trypanosome Infections*

Among the trypanosome species, as has been already indicated, there are some that are pathogenic and others that are non-pathogenic. A comparison of the resistance acquired by a host against the pathogenic forms and by a rat against the non-pathogenic *T. lewisi* is very illuminating. In the first case, the host either acquires no resistance (mouse) or periodically forms a trypanolysin (guinea-pig, dog, etc.) which is never permanently effective because it does not kill all the parasites, and those which remain eventually make good the periodical depopulations. In the second case, the host first produces an antibody which completely inhibits reproduction (cell-division) in the parasites, and thereafter each number crisis is so much gained by the host, since the cells that remain are incapable of repopulating the blood. It seems possible, then, that whether or not a given species is pathogenic depends upon the reaction of the host and that the formation of a reproduction-inhibiting antibody, in conjunction with some trypanocidal mechanism, is the immunological basis of non-pathogenicity.

Certain experimental findings bear out this hypothesis. Thus, there is evidence that when a rat does not form the reproduction-inhibiting reaction product in infections with *T. lewisi* the parasite produces a lethal infection. W. H. Brown (1914 and 1914 b) studied a strain of *T. lewisi* which was pathogenic to rats; the data in his second paper make it seem probable that what he considered the chief anomalies were due to the fact that the parasites reproduced longer than normally. Similarly, the author has observed apparently lethal infections of *T. lewisi* in which reproduction of the parasites was never completely inhibited. In some of the splenectomized rats of Regendanz and Kikuth (1927), in which no reproduction-inhibiting reaction product was formed, the infection progressed steadily until the death of the host—in other words, the infection resembled an infection with a pathogenic species in the mouse.

It would be interesting if the reverse process could be studied, viz., pathogenic trypanosomes in non-lethal infections. It is true that such infections exist but unfortunately cannot be thoroughly studied because of the scarcity of parasites in the blood-stream. For example, the pathogenic trypanosomes as a rule are lethal to most domestic and laboratory animals. Sheep, goats and cattle are notable exceptions in that although susceptible they generally exhibit a very light, but extended, infection, eventually recover and are then immune.

Laveran (1911) has given an extremely useful summary of his experiments with sheep and goats. During the infection, the trypanosomes were so scarce that they could not be found in the blood by microscopical examination but could be demonstrated by infection resulting in mice from the inoculation of blood from the sheep or goat. Such an infection might persist for from one to several months, and in one goat lasted for twenty-one months. The serum of such animals possesses a very potent protective and curative action (see pages 157 to 162) on the passage strain of trypanosomes originally inoculated; but this alone cannot explain the non-pathogenicity, since trypanosomes in the blood have been shown to become resistant to such properties. Because of the scarcity of parasites, however, it is impossible to ascertain whether the rate of reproduction is altered or not. Furthermore, there is some evidence that the trypanosomes that are fatal to man and domesticated animals produce no observable symptoms in the antelope and other wild game of Africa. These have so far been inaccessible for this type of analysis, but some of them may prove suitable and eventually become available. In this connection it is interesting to note that van Saceghem (1923), in trying to explain the non-pathenogenicity of certain African trypanosomes, postulated a reproduction-inhibiting immune property. As yet, however, such an immune property has not been demonstrated by suitable immunological experiments as the author and others have done in the case of *T. lewisi* infections.

VI. *Parasiticidal Mechanisms Other Than Lysins in the Trypanosome Infections*

1. PHAGOCYTOSIS

In the preceding discussion the major parasiticidal mechanisms have been trypanolysins and, with the exception of *T. lewisi* infections, no mention has been made of phagocytosis. There are, however, frequent reports in the literature in regard to phagocytosis, and it is necessary to consider them in detail as from their very nature they must belong to the parasiticidal agents which kill the organisms, but which do not inhibit reproduction *per se*. (See Figure 15.)

The process of phagocytosis has been observed both in the circulating leucocytes (chiefly in the large mononuclears, although to some extent in the microphages) and in the fixed tissue phagocytes. In regard to the blood leucocytes, the first observation was probably that of Laveran and Mesnil (1901), where living *T. lewisi* were

injected into the peritoneal cavity of immune rats. Levaditi and Sevin (1905) noted the process in animals having a natural immunity to *T. paddæ*. Mesnil and Brimont (1908 and 1909) described the same process in their study of the protective property of serum from infected animals. They found, after injecting immune serum and *T. brucei* intraperitoneally into a mouse and removing some of the

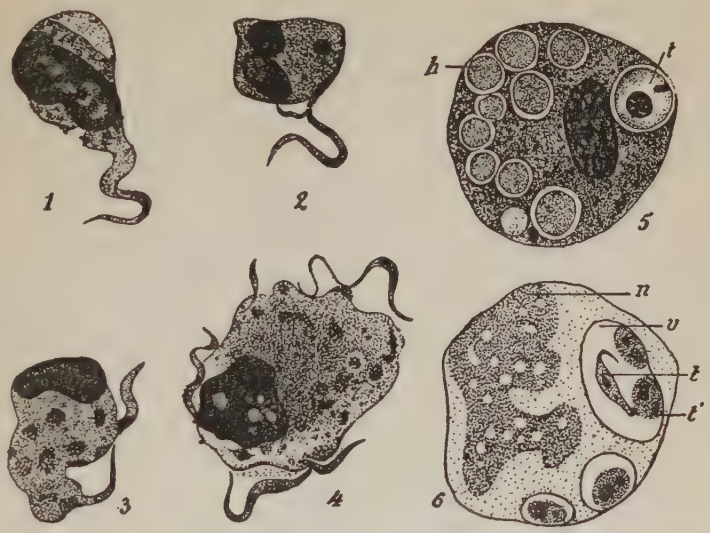


FIG. 15.—Phagocytosis of trypanosomes as seen in stained preparations. 1-4, phagocytosis of *T. togolense* (*T. brucei*) in peritoneal cavity of a mouse which had received protective serum; some trypanosomes are attached to leucocytes, some are partially engulfed, and some have been partially digested, so that only nuclear debris is visible within the phagocyte; 5, phagocytosis of *T. lewisi* in the rat; *h*, erythrocyte, *t*, trypanosome; 6, phagocytosis of *T. inopinatum* in the frog; *n*, nucleus of leucocyte, *v*, digestive vacuole containing an intact parasite (*t*) and two rounded forms (*t'*). (All figures from Laveran and Mesnil, 1912; 1-4 after Mesnil and Brimont; 6 after Brumpt.)

fluid at intervals for examination, that the leucocytes, mostly mononuclears, which increased enormously in numbers, actively ingested the parasites. The parasite at first seemed stuck to the leucocyte, generally, although not invariably, by its posterior, non-flagellated end; it was then engulfed by the very active and rapid pseudopod formation of the leucocyte and there quickly disintegrated. The process reached its maximum in the mouse within ten to thirty minutes. Examination of the mesentery, spleen, and blood showed that the destruction of the organisms was localized to the peritoneum.

Later, Levaditi and Mutermilch (1910) studied the mechanism very carefully *in vitro*. They ascertained that guinea-pig leucocytes (peritoneal exudate) plus inactivated serum from a normal guinea-pig did not affect the trypanosomes *in vitro*, whereas leucocytes plus inactivated serum from an infected guinea-pig bled at the crisis had the power of attacking the parasites and then ingesting them. If, however, the parasites were resistant to the serum, phagocytosis did not occur. They divided the process into two phases: (1) that of attachment of the organism to be phagocyted to the leucocyte, and (2) that of actual incorporation. In support of this they found that the attachment of sensitized trypanosomes alone, took place if the *in vitro* mixture was kept at 0° C. and also if the leucocytes had been previously killed by a three-day sojourn in the ice-box, by heating to 45-60° C., by successive freezing and thawing, or by mechanical action. This phase took place upon the chance meeting of phagocyte and parasite, and since it required no active intervention on the part of the phagocyte, the authors believed it to be a physico-chemical phenomenon. It was, however, specific, because only sensitized trypanosomes (in the presence of the antibody or after fixing the antibody) attached themselves to (and only to) leucocytes. The second phase, the ingestion phase, they believed to be a vital phenomenon for it could be prevented by an injury to the phagocytes. During the attachment stage and until the parasite was partly engulfed, the parasite was in violent motion. The whole process consumed about five minutes. Roudsky (1911) and Delanoë (1911 and 1912) confirmed the *in vivo* occurrence of phagocytosis of *T. lewisi* in the mouse. The latter found that phagocytosis took place not only in the peritoneum but throughout the body.

In regard to the rôle of the fixed macrophages, Sauerbeck, as early as 1905, in a study of *T. brucei* infections in rats, guinea-pigs, rabbits and dogs, maintained that the chief pathological picture of the disease was a stimulation of the macrophage system and that within these cells parasites could be found in various stages of digestion. (Cf. Neporojny and Yakimoff, 1904; Yakimoff, 1908; and Massaglia, 1909.)

Throughout the literature various degrees of importance have been ascribed to phagocytosis and lysis: some authors have maintained that one or the other is the sole method of defense; some that they share equal honors, and some that, although lysis is the fundamental mechanism, phagocytosis is responsible for clearing up the debris, etc. No one can doubt the occurrence of phagocytosis after its careful description by so many competent observers, but the

present author after many attempts has been unable to observe the process. These discrepancies may simply be due to the fact that the two processes, as has been suggested in Chapter I, are in reality expressions of the same process, and the occurrence of one rather than the other may depend on such things as the experimental conditions, titer of the immune serum, etc. The work of Levaditi and Mutermilch strongly suggests that at least the first stage of phagocytosis, i.e., "attachment," is a characteristic of lytic serum and follows the same rules of strain specificity as lysins. If this be true, a lytic serum of high titer may destroy the parasites before the phagocytes ingest them, and if of low titer, the converse may occur. Depending on the titer of lytic serum, then, the phagocytes may ingest seemingly normal parasites, in other cases immobilized parasites, in others moribund ones, and in still others the debris from completely disintegrated ones. In thus considering lysis and phagocytosis as expressions of the same mechanism, I am again emphasizing the view that there is no fundamental distinction between humoral and cellular phases of immunity, but that both are fundamentally expressions of the cellular reaction of the host.

2. CHANGES IN BLOOD SUGAR

Recently considerable interest has been elicited in the changes in blood sugar during the course of different protozoan infections, and as will be seen later in discussing malaria, some authors feel that a diminution in blood sugar may be followed by the destruction of the parasites. No attempt will be made to review all of the work on blood sugar in trypanosomiasis, but especially pertinent is the work of Schern (1925). In cover-slip preparations of the blood of infected rats, trypanosomes remained active for several hours if taken at the beginning of the infection, but became motionless in about ten minutes if taken a few hours before death. In fact, in this way, the probable survival time of a host could be forecast. Furthermore, the trypanosomes from the terminal stages of an infection could be reanimated by the addition of dextrose, levulose, or a thermostabile fermentable constituent of normal serum or liver extract. The reanimating property of liver could be decreased by treatment with insulin. Additional evidence for these general conclusions has been furnished by Bruynoghe, Dubois and Bouckaert (1927), Regendanz and Tropp (1927) and R. Knowles and Das Gupta (1928). In trypanosome infections there is evidence that carbohydrates, such as sugars, are necessary for motility (which is probably a delicate

measure of vitality), and that these disappear from the liver and blood as the infection progresses.

Just how important their disappearance is in the destruction of the parasites in the animal body is unknown, but certainly trypanocidal antibodies play the major rôle during crises. In fact, the decrease in the carbohydrates probably takes place not only in those infections where number crises occur, but also in infections in rats and mice where a continuous uninterrupted increase of the parasites occurs (cf. the original work of Schern). If there is any *in vivo* destruction of the parasites by a hypoglycæmia of the host, it is independent of the antibody destruction, and to the present author it would seem that although the decrease in blood sugars during the infection would directly influence the survival time outside of the body, within the body the parasites would not be actually killed by hypoglycæmia of the host until the host itself was killed.

For a discussion of the effect of thyroid feeding and thyroidectomy, both of which may produce their effect by influencing the blood sugars, the reader is referred to the very interesting work of Knowles and Das Gupta (1928) on infections with *T. evansi*. These authors found that in monkeys subtotal thyroidectomy tended to make the infection more acute, and some indication was obtained in rats that intensive thyroid feeding produced the opposite effect.

VII. *Malarial Infections*

Although considerable work has been done on the serology of malaria in man (see Chapter II), investigations on the course of uninfluenced infections with the three species of human malaria have been greatly handicapped because humanitarian reasons preclude extensive work on human subjects and ordinary laboratory animals are not susceptible to the infections. With the advent of the use of malaria in the treatment of paresis many advances have been made, but even so, most of our knowledge has been gained from the closely related malarial parasites of birds.

The avian parasite * occurs within the red cells in its vertebrate

* Until recently the avian parasites used had been known under the single name of *Plasmodium* or *Proteosoma præcox*, but recently Hartman (1927 b) has maintained that sparrows are in reality infected by three species, *Plasmodium præcox*, *P. cathemerium*, and *P. inconstans*. The work of American investigators reviewed in this chapter was done on two strains: Whitmore, Ben Harel and Boyd used Whitmore's strain which, according to Hartman, is *P. præcox;* and Hartman, L. G. Taliaferro and the author have largely used Hartman's strain, which is *P. cathemerium*.

host where it exhibits two types of development, the asexual and sexual (Figure 8). The asexual cycle, because it is the characteristically vertebrate part, is the most important from the present point of view. In general, the course of infection is as follows: After a parasitological incubation period (1) during which no organisms can be found, the parasites rapidly increase in the blood (2, acute period) until sometimes every other cell is parasitized; then if the bird does not die, there is a crisis (3) when most of the parasites are killed; but although some may remain for a week or more (4,

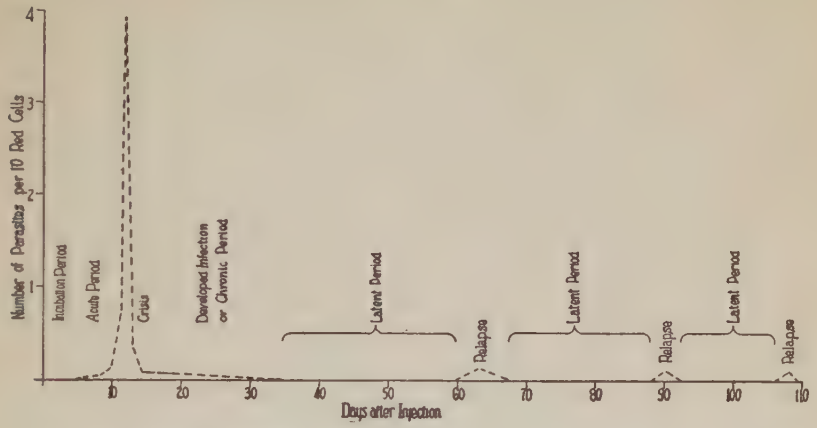


FIG. 16.—Graph illustrating the type of number curve usually encountered in the course of a malarial infection in the canary. (From the author, 1926.)

chronic period *), in time all the parasites apparently disappear from the blood (5, latent period). Thereafter, if the bird's resistance is lowered in any way, they may reappear for a week or more in secondary acute rises and crises (6, relapse). (See Figure 16.)

The exact enumerative studies made by the Sergents (see especially 1918), Ben Harel (1923), L. G. Taliaferro (1925), Boyd (1925) and Hartman (1927) all indicate that the outline just given is the general type of infection encountered in birds.† There may be, however, great variations in the lengths of the different periods and

* As this term is used to signify a different thing in human malaria, another term, the period of developed infection, is sometimes used for this period of the infection.

† Compare the similar results of Senevet and Witas (1922 and 1922 b) on the *Hæmoproteus* of pigeons.

in the severity of infection during those periods in which the parasites can be demonstrated in the blood. The "extended irregular infections," which Ben Harel described, were probably cases in which a series of relapses were superimposed on the usual acute rise and chronic portion of the infection, and possibly may have been due to the daily removal of blood samples for erythrocyte counts.

Each of the periods may now be considered in turn. All evidence is consistent in indicating that the host *acquires* no resistance either during the incubation period or during the acute rise of the infection, but that the parasites reproduce at a uniform rate and that a constant number of them survive. As most of the work bears on the acute rise of the infection, it will be considered first. During the acute rise of the infection L. G. Taliaferro (1925), by the method previously described, found that the rate of reproduction of the parasites was constant (the length of the asexual cycle was twenty-four hours throughout in one strain*) and that the parasites increased in the blood according to a geometrical progression, as would be expected if the host acquired no resistance. She found, however, that out of an average of 15.5 merozoites produced by each mature schizont (aside from the few gametocytes formed) approximately ten die or at least do not become mature schizonts. The constant rate of increase during the acute period has been verified by Hartman (1927), who ascertained, however, that the actual rate may vary considerably among different birds. Hartman also made a careful study of the rate of death of the parasites which perish between each asexual generation, and showed that the rate of death is a constant for twenty-one hours of the twenty-four period. Furthermore, although not stressed in his paper, his data check the approximate number of parasites which perish as found by L. G. Taliaferro (1925). Thus, he found that within twenty or twenty-one hours of the twenty-four hours between two sporulation periods in bird 203, the parasites decreased from 1,843 to 602 per 10,000 red cells; in bird 282, the parasites decreased from 527 to 258 per 10,000 red cells; and in bird 297 the parasites decreased from 1,950 to 921† per 10,000 red cells—figures which represent a decrease in the parasites of 67, 51, and 53 per cent, respectively. As pointed out by L. G. Taliaferro, this non-viability of the majority of parasites produced by each sporulation probably represents the

*Drensky and Hegner (1926), Hartman (1927), L. G. Taliaferro (1928) and Boyd (1929) have corroborated the occurrence of the cycle.

† In these figures I have taken the computed values after they were fitted to a smooth curve, rather than the actual observed counts.

suitability of the bird as a culture medium for the parasites and is not in any sense an acquired resistance. Evidence that there is similarly no resistance acquired by the host during the incubation period is purely inferential, as the parasites are not in the blood. It is significant, however, that Boyd (1925) found a definite correlation between the number of parasites injected into the bird and the length of the incubation period (-0.522 ± 0.053) and the number of parasites at the peak of the infection (0.340 ± 0.074). This is just what would be expected if the rate of reproduction and the rate of accumulation of parasites are constant throughout and indicates

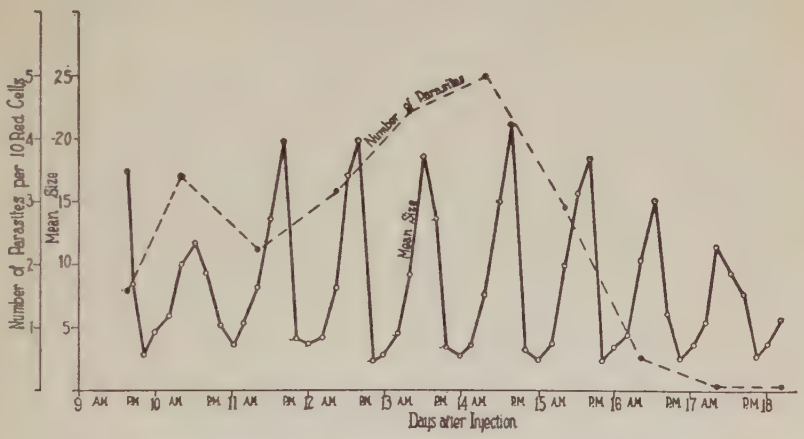


FIG. 17.—Graph showing the changes in mean size of the asexual forms and the number curve through the acute period and crisis in an infection with *P. cathemerium* in a canary. The uniform rate of reproduction (as shown by the regularity in the mean-size curve) and the type of number curve (as seen in this and the preceding figure) indicate that there is a parasiticidal but no reproduction-inhibiting resistance developed. (From data by L. G. Taliaferro, 1925.)

that the incubation period is simply the time it takes the parasites to become numerous enough to be found in the blood.

During the remainder of the infection, there is a wholesale destruction of the parasites at the crises which terminate the acute, chronic and relapse periods (Figures 16 and 17). Moreover, the author and L. G. Taliaferro (1929 and 1929 b) have shown that if a large number of washed parasitized cells are injected intravenously into an infected canary during the latent period, they are quickly removed from the circulation (probably by the phagocytes), whereas in a normal canary they are not removed but steadily increase in

numbers until the death of the canary or until a crisis ensues (Figure 18). One very interesting feature in this work is that the parasites are being removed from the peripheral blood continuously —a fact which is incompatible with Bass and John's (1912) assumption that the greatest hazard is when the parasite is passing from one cell to another, but agrees with the findings of Hartman (1927) for the death of the parasites during the acute infection.

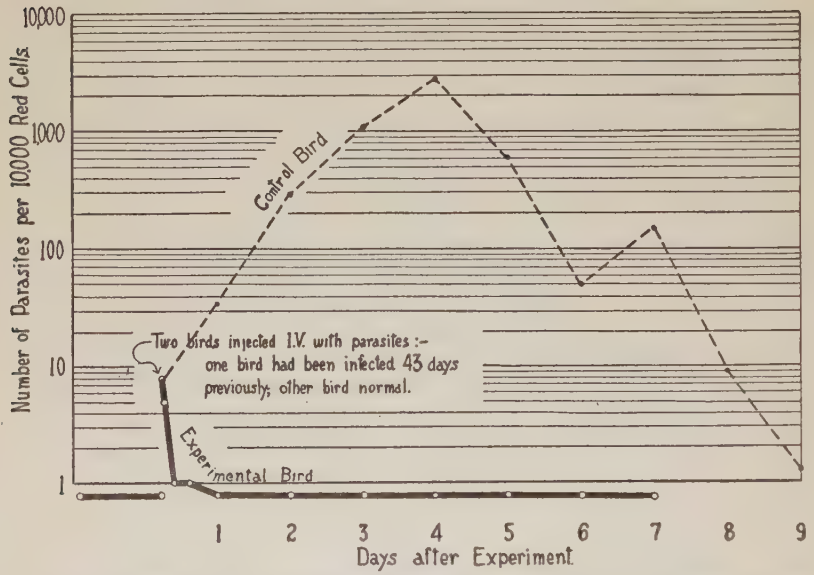


Fig. 18.—Experiment showing the disappearance of washed parasitized cells from the blood of a bird in the latent infection (experimental bird) and the survival of the same type of cells in an uninfected bird (control bird). Both birds were injected intravenously with enough washed infected cells so that a few minutes later both showed an infection of about eight parasites per 10,000 red cells. In the bird with the latent infection, these were removed from the circulation within twelve hours, but in the uninfected bird they survived and progressively increased in numbers, initiating a typical infection. (From the author and L. G. Taliaferro, 1929.)

The destruction of the parasites is not associated with a retardation of the rate of reproduction of the parasites, according to L. G. Taliaferro (1925). She found that the asexual cycle, which is a measurement of the rate of reproduction, as explained previously, takes the same time (twenty-four hours in one strain) throughout the acute, chronic, and relapse periods (this is shown for the acute period and crisis in Figure 17), and that each stage in the cycle takes

place at exactly the same time in the relapse as it had in the acute and chronic periods.

The malarial infection in birds, then, may be expressed in terms of resistance, according to L. G. Taliaferro, as follows:

"After the incubation period the asexual stages of the parasites are to be found in the peripheral blood in varying numbers during the entire course of the infection and undergo their cycle of development and reproduction at the same rate throughout. From the very beginning, only a number of the merozoites are viable; this probably represents a natural resistance of the host. During the first part of the infection relatively few parasites are killed, so that they accumulate in the blood and give rise to the acute stage of the infection. Sooner or later, however, as the acquired resistance is built up, a large proportion of the parasites are killed. There may then be a temporary relapse, but eventually, the destruction of the parasites equalizes or exceeds the number produced by reproduction, and the chronic period or low grade of blood infection ensues. In time, the destruction becomes so great that no parasites can be found in the blood (latent period), but their presence can be demonstrated by Whitmore's technique [i.e., by injecting blood into fresh, uninfected canaries]. This continues until some condition, such as the injection of adrenalin, temporarily stops the destruction and the parasites accumulate again, causing a relapse."

From the very precise enumerative studies of Ross and D. Thomson (1910b) on human malaria and from the general observations of other workers, it seems probable that the course of infections of both *P. vivax,* the benign tertian parasite, and *P. malariæ,* the quartan parasite, is very similar to that of avian malaria. On the other hand, it is difficult to analyze the effects of resistance in *P. falciparum,* the parasite of malignant tertian fever, as well as of certain avian malarial parasites, because schizogony does not occur in the peripheral blood.

From the preceding discussion, two parasiticidal mechanisms—as evidenced by the constant death-rate of most of the progeny from each asexual generation during the acute period, and by the very much increased death-rate terminating the acute period and continuing throughout the remainder of the infection, except during the acute rise in the relapses—seem to be operative during the course of malarial infections. The first is not an acquired resistance in any sense; the second is. At present, however, it is not clear whether the acquired parasiticidal mechanism is simply an enhancement of the preëxistent and already operative mechanism present in normal birds, or whether it is different from and superimposed on the natural mechanism. It is true that the data, reviewed on page 232, strongly suggest that the wandering mononuclears and fixed tissue phagocytes

ingest the parasites at the crisis and during the latent infection; but experimental evidence has not ascertained why, in such experiments as carried out by the author and L. G. Taliaferro (1929), the phagocytes of the bird with a latent infection (i.e., acquired immunity) should ingest the organisms, whereas those of the normal bird do not, or do so only to such a limited extent. There are at least four possibilities. Some antibody mechanism may render the parasite or parasite red cell combination phagocytable (viz., an endothelial opsonin), or it may kill or lyse the parasites so that the phagocytes ingest them in various stages of vitality or even as fragments. Some of the simpler serum constituents of the blood may so injure the parasites that they become moribund and disintegrate, whereupon the phagocytes remove them. The phagocytes themselves may become changed (variously expressed as "an education of the phagocytes," an attuning, etc.) so as to ingest more parasites. Or, finally, the reticulo-endothelial system as a whole may be greatly augmented, and result in an increase of non-specific phagocytosis. These possibilities had best be considered in detail.

All attempts to show that removal of the parasites is associated with an antibody have failed. Thus, Moldovan (1912) failed to obtain any evidence of protective or complement-fixing antibodies in the avian infection and the author and L. G. Taliaferro (1929 b) have been unable either to sensitize infected red cells with serum from infected birds in the latent period or to obtain a passive transfer of a lethal factor. This might be taken to indicate that the parasites simply injure the red cells to such a degree that they are phagocyted, but such an assumption is not tenable because the same infected cells are not phagocyted by the normal uninfected bird. The failure to find an antibody basis for the destruction of the parasites in birds suggests the same lack of antibody basis in man, but one hesitates to draw too close a parallelism because no anti-malarial antibodies of any type have been found in the bird, whereas they have been found in man.

The recent papers of Hegner and MacDougall (1926) and MacDougall (1927) suggest that the factor which kills the parasites or which makes the parasite red cell combination phagocytable is connected with the simpler serum constituents. These workers have found that increasing the blood sugar by feeding solutions of glucose brings about conditions favorable to the accumulation of the parasites in the blood, whereas decreasing the blood sugar by injecting insulin probably inhibits the accumulation of the parasites. Their experiments are in accord with the work of Bass and Johns (1912)

and others who have found sugar necessary for the cultivation of the malarial organisms outside of the body and with the results of Bass and Johns (1913) who succeeded in cultivating the parasites in the blood from a case of diabetes without the addition of sugar.

The results on the relation of sugar to human infections are rather inconclusive. Working with induced benign tertian malaria in general paresis, Rudolf and Marsh (1927) found glycosuria in a higher percentage of cases of general paresis treated with malaria than untreated. (See their paper also for a review of previous work on the same subject.) They found, too, that the blood sugar during induced malaria varied inversely as the temperature, although it might be higher after the fall in temperature than before pyrexia. Administration of glucose was ineffective against objective symptoms and parasites, but apparently relieved subjective symptoms. Administration of insulin produced indefinite results, but in 60 per cent of the cases the fever terminated after administration, and the relapses which followed were of a lower degree of pyrexia than those following quinine therapy. From these data they concluded that lowering the blood sugar did not appear to be the cause of the cessation of the fever.

In all this work there is the possibility that a diminution of the blood sugar is a concomitant change rather than the basis for the disappearance of the parasites. Thus, in the trypanosome infections, where the death of the parasites at each number crisis in the infection is directly related to the production of antibodies, blood sugar changes have been observed quite similar to those described for avian malaria (see page 133). In any case a great deal more work has to be done before the acquired immunity of birds to malaria can be associated solely with changes in blood sugar.

In regard to the possibility of the acquired resistance being associated entirely with changes in the phagocytes or an increase in their number, there is no conclusive evidence, although this problem is now being studied in conjunction with Dr. P. R. Cannon of the University of Chicago.

The preceding discussion leads directly to a consideration of the mechanism of relapse.

Infections with malaria, whether they be in man or birds, are characterized by their tendency to relapse. Many workers on human malaria divide these relapses into two types: true relapses which appear after short intervals, and recurrences which appear after long intervals. In spite of a long list of investigations there is no unanimity of opinion as to how the malarial parasite subsists in the body

during the latent periods between relapses. The more important hypotheses may be classified under three heads:

1. Some of the female gametocytes were supposed to survive the action of the quinine or the host's resistance and at the beginning of a relapse to be stimulated to undergo segmentation, which again would initiate the asexual cycle of the parasite. This hypothesis of so-called "parthenogenesis" of the female gametocyte was advanced by Grassi (1900) and Schaudinn (1902) and has been supported by many subsequent observers. Schaudinn depicted in detail what he believed to be stages in the process. In an able critique of this hypothesis, and in particular, of Schaudinn's work. J. D. Thomson (1917) pointed out the following: (a) Schaudinn overlooked the fact that in all known cases of parthenogenesis the unfertilized female gamete exhibits essentially the same general course of development pursued by the fertilized gamete. Therefore, if there were a parthenogenetic development of the female gametocyte, it should show many of the stages peculiar to the mosquito host and not give rise directly to asexual segmenting stages. (b) All of Schaudinn's figures depicting the female gametocyte undergoing segmentation can be easily interpreted as cases in which the red cell contains a female gametocyte juxtaposed to an ordinary segmenting schizont. In fact, Thomson found many such stages in his own preparations. Although by no means conclusive, another bit of evidence against parthenogenesis of the gametocytes is the fact that in those species of malarial organisms in which only gametocytes occur in the peripheral blood, the blood is not infective to a new vertebrate host. Similarly, in the use of therapeutic malaria in the treatment of paresis, blood containing only gametocytes has been found not to be infective (see Mühlens, Weygandt and Kirschbaum, 1920, and Weber, 1923). If the gametocytes could start a new asexual generation, it would appear probable that they could do so under such conditions. In short, there is little evidence to support the so-called parthenogenetic view.

2. Specialized, resistant, asexual forms, which were supposed to survive the periods of latency until conditions were again favorable in the blood for them to renew their asexual schizogony, form an integral part of the hypotheses advanced by such investigators as Celli (1900), S. P. James (1917 and 1920) and Craig. In the particular view advanced by Craig (1906, 1907 and 1926), two young hyaline rings were supposed to conjugate within the cell and the resulting zygote was supposed to grow until it filled the red cell. It then left the red cell and was carried by the blood-stream to the spleen and bone marrow where it rested until proper stimuli caused

it to liberate young forms which renewed the asexual cycle. Craig (1926) still holds this view for relapses occurring at long intervals, but accepts the hypothesis of Ross and others, given later, for relapses at short intervals. In spite of Craig's description of conjugation and a similar description by Ewing (1901), many biological objections preclude the supposition that merozoites destined ordinarily to continue the asexual cycle should assume the rôle of gametes. Furthermore, the process is difficult to demonstrate because the close approximation of two forms within the cell can easily be mistaken for conjugation. The resistant forms described by James are asexual forms which remain quiescent until stimulated by favorable conditions to reinitiate the asexual cycle. For a discussion favoring this view, as well as illustrations, the reader is referred to J. D. Thomson and Woodcock (1922).

3. Ross early advanced the hypothesis that the malarial parasites continue their normal asexual cycle throughout the infection, but that their vitality is lowered during the latent periods and renewed during relapses. (For a discussion of this view, see Ross, 1910.) Bignami (1910), W. M. James (1913) and Whitmore (1918) hold essentially similar views. The asexual parasites during the latent period, according to Bignami, become resistant to quinine or antibodies just as trypanosomes are known to do.

In the author's opinion the studies on human and avian infections support Ross's assumptions. It seems probable that, not only is the asexual cycle continuous during the latent period, but continuous at the same rate (vitality) as during the acute rise; and the scarcity of the parasites is accounted for by the killing mechanism developed by the host, which is removed in some way during relapse. Some of the chief evidence in favor of Ross's hypothesis may be summarized as follows:

1. Ross and D. Thomson (1910) in their extremely careful enumerative studies of human malarial infections showed that the general trend of the number curves strongly indicated that the asexual parasites persisted during relapses of short duration, but were too few to be found in blood films. Many investigators who postulate resistant forms for the production of relapses after long intervals follow Ross and Thomson's conclusions for relapses after short intervals.

2. Working with avian malaria Whitmore (1918) found that throughout the latent period, although no parasites could be found, the blood was infective to other birds for as long as twenty-nine months after infection. As the asexual parasites are the only ones

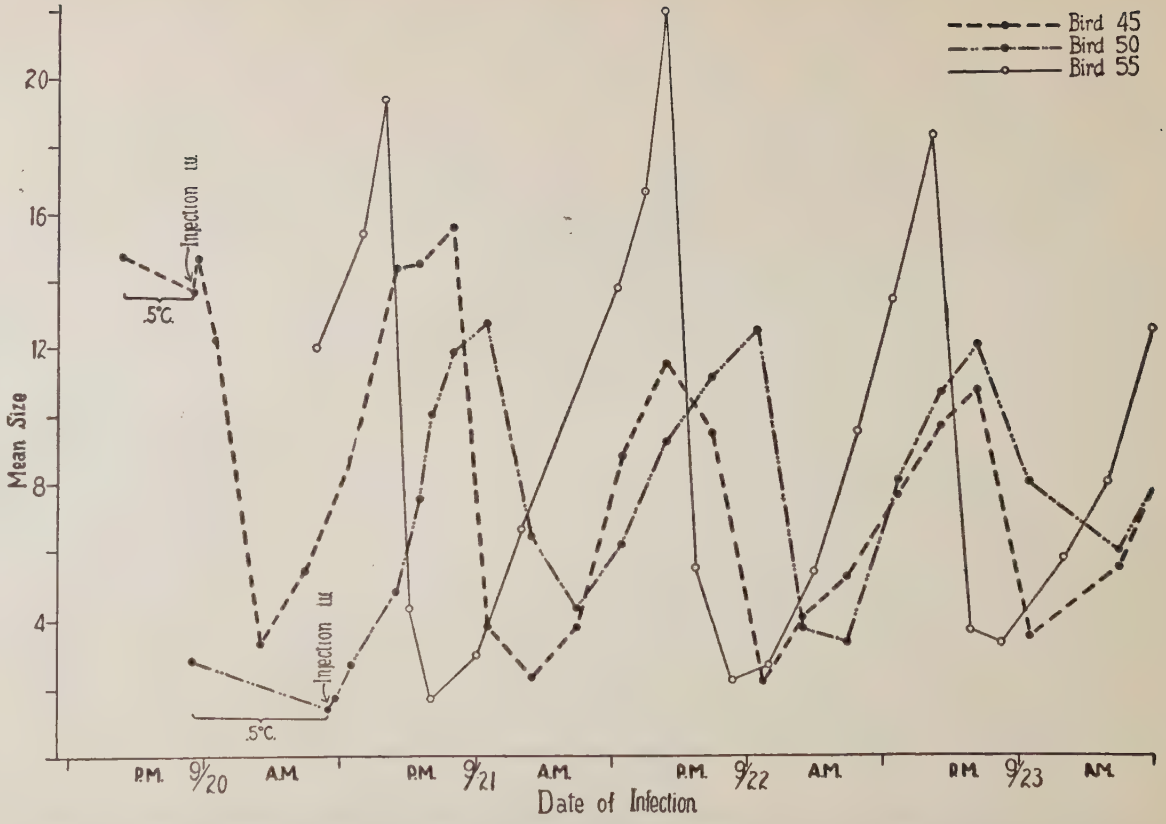


FIG. 19.—Graph showing cycles of growth and sporulation of parasites, after refrigeration *in vitro*, as represented by the mean size of fifty parasites at four-hour intervals, and for comparative purposes, cycles from a normal infection. The parasites in bird 45 had been refrigerated *in vitro* for six hours; those in bird 50 had been refrigerated for twelve hours; and those in bird 55 were normal. Note that the cycles in birds 45 and 50 are at first delayed, but gradually, by a speeding-up of the process of growth and sporulation, more closely approximate those of the control bird 55. Intravenous injection, hence immediate infection and actual dates. (From L. G. Taliaferro, 1928.)

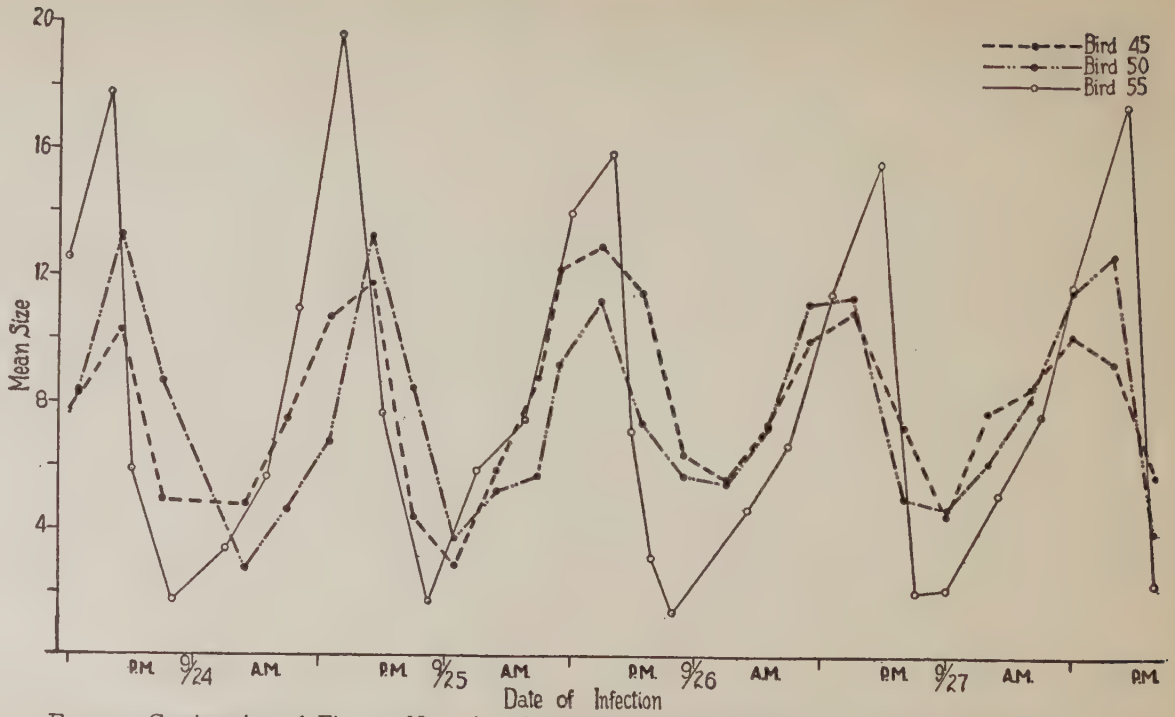


FIG. 20.—Continuation of Fig. 19. Note that the cycle in the experimental birds by the ninth day closely corresponds in time to that of the control (bird 55). (From L. G. Taliaferro, 1928.)

which are known to infect other hosts, he quite justifiably assumed that a few were present in the peripheral blood throughout the latent period. Mazza (1924) has found birds infective four years and two months after infection.

3. Ben Harel (1923) after patient search was actually able to find asexual parasites in the process of asexual reproduction during latency.

4. L. G. Taliaferro (1925) showed that whenever the parasites were found in the blood not only was asexual reproduction occurring, *but at the same rate* as during the acute rise of the infection. At the time of her paper she considered it significant that when they reappeared during a relapse the time of each stage in the asexual cycle was exactly what would be expected if it had occurred uninterruptedly throughout the latent period. This last evidence would be conclusive except that the asexual cycle is diurnal, and as she (1928) showed later, is under some regulatory device which apparently causes the cycles to take place at the same time. Thus, when infected blood, which had been refrigerated six or twelve hours, thereby delaying the cycle, was injected into normal birds, the parasites took a shorter time each day to complete their asexual cycle until they had "made up" the lost time and were again dividing and sporulating at the original time. (See Figures 19, 20.) As she pointed out, these data can be interpreted in two ways: To those who believe the parasites lie dormant during latency, this would mean that no matter when a new series of asexual cycles was initiated at the beginning of relapse, the parasites would within a few days exhibit the usual time intervals in their cycle; whereas to those who believe that the asexual cycle continues uninterruptedly throughout latency, this would indicate how difficult it is to modify the asexual cycle in any way. By the ingenious method of keeping infected birds in the dark during the day and in the light during the night, Boyd (1929) found that the sporulation time shifted within three days from the evening to the corresponding morning hours. This evidence, as he pointed out, indicates that the periodicity of the cycle is influenced by the host.

To the present author, the available experimental work indicates that in certain respects relapse in malaria is similar to relapse in the infections with the pathogenic trypanosomes, but in other respects is markedly different. In both types of infection the parasites apparently reproduce throughout the infection, both when they are present in the blood and during the so-called latent periods, at a high and more or less constant rate, and their disappearance from the blood

is associated with a parasiticidal mechanism which does not affect reproduction *per se*. The reaccumulation of the parasites in the blood during relapses seems, however, to be due to different causes. Among the pathogenic trypanosomes it is associated with the fact that the parasites which survive the parasiticidal mechanism become resistant to it, thus allowing a repopulation of the blood-stream, whereas among the malarial infections, it is associated with a temporary removal of the parasiticidal mechanism.

VIII. *Summary*

The study of various protozoan infections, all the forms of which occur in the peripheral blood where they are accessible for study, reveals the fact that a resistance may be acquired by the host which is directed toward an inhibition of the rate of reproduction of the parasites or toward their destruction after they are formed. By suitable methods which have been devised to differentiate these two factors, the following types of infections have been established:

When the so-called pathogenic trypanosomes are grown in the mouse (sometimes in the rat), the host acquires no resistance affecting either the rate of reproduction of the parasites or their destruction after they are formed, and is killed in a comparatively short time.

When the same pathogenic trypanosomes are grown in the guinea-pig, rabbit, dog, and sometimes the rat, the host periodically acquires a trypanolytic property in its serum which destroys large numbers of parasites after they are formed; but it is eventually killed because the few parasites that remain after each trypanolytic crisis continue their reproduction and become biologically altered so that they are no longer susceptible to the trypanolysin and can thus repopulate the blood. When the same pathogenic trypanosomes are grown in goats, sheep and cattle, the host also acquires a trypanocidal property in its serum which destroys such large numbers of the parasites that the infection is of a very low grade and the host recovers in several months. Concomitant factors responsible for the non-pathogenicity of the parasites are not clear.

When the non-pathogenic *T. lewisi* is grown in the rat and guinea-pig, the host acquires a reproduction-inhibiting property in its serum, which inhibits the reproduction of the parasites, and a trypanolytic property, which destroys them after they are formed, with possibly an additional phagocytic activity. Consequently, since the rate of reproduction of the parasites is inhibited early in the infection (tenth

day), the parasites which survive a first trypanolytic crisis cannot repopulate the blood but are eventually destroyed, and the host recovers from its infection in several weeks to several months.

When the usually non-pathogenic malarial parasite is grown in birds, the host acquires a parasiticidal mechanism which is probably connected with a heightened phagocytic activity and may be associated with a diminution in blood sugar. Since the rate of reproduction of the parasites is not altered and since the phagocytic activity does not seem able to exterminate the infection, the parasites may persist for years. Throughout this period, they may reaccumulate (relapse) at any time provided conditions, adverse to the host, remove the parasiticidal mechanism.

CHAPTER IV

THE PROTECTIVE AND CURATIVE ACTION OF IMMUNE SERUMS

In the study of the effect of immune serum on the course of infections it is customary to differentiate sharply between protective and curative action. Experiments on protective action are carried out by introducing the immune serum approximately at the same time as the virus (generally simultaneously or not more than a day before or after), whereas experiments on curative action are carried out by introducing the immune serum after the infection has been well established, viz., in blood infections, when the parasites have made their appearance in the blood. It is obvious that such a distinction is arbitrary because one type of experiment grades into the other. It is, nevertheless, useful in practice. Moreover, since a given immune serum may quite frequently be protective but not curative, some authors have considered that the two are based on different principles of the serum. This distinction, however, does not seem to be borne out by some recent work of the author and T. L. Johnson which indicates that the apparent difference may be due to the ratio of the amount of virus to the dose of serum.

I. *Protozoan Infections*

1. TRYPANOSOMIASIS

1. *Protective property of immune serum.* In some of the protozoan infections, notably those of trypanosome origin, it has been found that serum taken from an animal during a chronic or even subacute type of infection or after recovery or treatment, protects an animal against infection, to a greater or less degree, when injected simultaneously with the same species of organism. This "protection" is indicated either by a complete inhibition of the infection or by a longer survival time for the experimental animals, as compared with controls infected at the same time with the same dose of virus but without the protective serum.

Probably the first observation of this phenomenon was made by Rouget (1896), who found that the serum of rabbits and dogs,

which were infected with the pathogenic *Trypanosoma equiperdum* and had become cachectic, exerted a protective action in a dose of ⅓ c.c. in mice infected with the same trypanosome, as measured by their survival time. The same results were obtained whether a mixture of virus and protective serum was injected or whether the two were injected into different parts of the body. When the protective serum was injected into mice after the trypanosomes had appeared in the blood, i.e., two or three days after infection, there was little, if any, effect on the length of survival. In other words, in these experiments, the protective serum did not possess curative properties.

A few years later, Rabinowitsch and Kempner (1899) found that 1.0 c.c. of immune homologous rat serum would protect rats against infection with the non-pathogenic *T. lewisi* when introduced simultaneously or a day before or after the virus, whereas various organ emulsions of immune *T. lewisi* rats and serum from a hamster infected with *T. rabinowitschi* failed to protect against *T. lewisi.* Laveran and Mesnil (1901) corroborated and greatly extended this work. Their immune serums were obtained from rats which, after having recovered from an infection with *T. lewisi,* were "hyperimmunized," i.e., reinjected several times with the virus. Such serums, when injected in intraperitoneal doses of about 0.5 c.c. simultaneously with *T. lewisi,* either prevented or greatly hindered the infection. Some were even efficacious in doses as small as 0.1 c.c. When injected a day before or after the trypanosomes, or at the same time but into a different part of the body from the organism, a dose of 1.0 c.c. was required. Such results led Laveran and Mesnil to hope that a sero-therapy might possibly be developed against pathogenic trypanosome infections, but so far this has not materialized.

In trying to realize this hope, Laveran and Mesnil in 1902 worked on the pathogenic *T. brucei.* They found that the serums of goats and sheep after recovery from nagana exerted a protective action on mice, when mixed with the virus and injected in comparatively large doses, viz., 1 c.c. Like Rouget, they found that the serums gained this property as the infection progressed but did not develop any curative property. A similar low grade protective property, as tested in mice, was found by Nocard (cited by Laveran and Mesnil, 1902) in cattle which had recovered from nagana. Laveran and Mesnil (1902 b) showed further that these immune serums from goats and sheep, although showing a protective property for the homologous trypanosome in mice, did not exert any protective action on a heterologous trypanosome, in this case *T. equinum;* but later

(1904) after cure, they found that one of the sheep had acquired this property.

These investigations were substantiated and supplemented by studies on various other pathogenic trypanosomes. Thus, Martini (1905) found that the serum of calves recovered from *T. togolense* (= *T. brucei*), when mixed with the passage virus and given in doses of 0.5 c.c., inhibited the infection in mice. The same results were obtained with dogs. In addition to this protective action he found that the serum also possessed agglutinating properties. Kleine and Möllers (1906) found that the serum of a donkey which had received several massive doses of the same strain would protect mice in doses of 0.5 c.c. if given a day before or a day later than the virus. It was not, however, effective against the relapse strain in the donkey nor against *T. gambiense*.

Franke (1905) found that the serum of a monkey (*Cercopithecus callitrichus*), which had been cured of mal de caderas (*T. equinum*) by a combined treatment with arsenicals and trypan red, possessed both agglutinating and trypanocidal properties against *T. equinum* but not against *T. brucei* or *T. evansi*. In addition, if 0.5 c.c. of this serum was mixed with its homologous virus, it prevented infection when injected into mice either at once or after standing two hours. On the contrary, the same procedure with heterologous trypanosomes, *T. evansi* and *T. brucei*, resulted in the usual course of infection. It is noteworthy that the recovered monkey was reinfected, but that its serum did not prevent the parasites isolated during the course of the infection from infecting mice, although it still prevented the parasites of the originally infective strain from infecting mice—a fact which confirmed the earlier views, viz., that trypanosomes could exist in the blood simultaneously with the protective properties. Thiroux (1906) likewise observed that serum from cases of sleeping sickness retarded infections of *T. gambiense* in mice.

At least one practical application of this protective property has been attempted. Diesing (1905) injected cattle with the serum from donkeys which had recovered and had been hyperimmunized, with the hope that these cattle could cross the region of the Kamerun which was heavily infested with tsetse flies. The first attempt, with two lots of twenty cattle each, was unsuccessful. A second attempt with 152 cattle gave better results. Of the animals which received 40-50 c.c. of serum from a donkey that had survived three to six inoculations of heavily infected blood, only a small percentage acquired any infection during the passage. Apparently the immunity did not last more than fifteen days.

A classical investigation of this property was carried out by Mesnil and Brimont (1909) working with *T. evansi, T. brucei* and *T. togolense* (= *T. brucei*). In one of their experiments, they found that the serum of a goat, infected with the *togolense* strain of *T. brucei* in doses as small as from 0.05 to 0.1 c.c., when left in contact with the virus two to four minutes and then injected, completely protected mice. After confirming previous work by ascertaining that a protective property was quickly acquired by serum of animals with a chronic or subacute infection, but very quickly lost after the animal had recovered from the infection, they found it possessed the following characteristics: 1. The serum of animals, exhibiting subacute and especially chronic infections possesses a protective property which arises very quickly, though somewhat variably, following infection, then diminishes following recovery. 2. This protective property resists heating for one half to three quarters of an hour at 56-64° C. (Of course, this does not remove it from the complement-amboceptor group of antibodies, because the tests were carried out *in vivo*.) 3. It is fixed, in part at least, by the trypanosomes. Thus, when trypanosomes which have been "sensitized" *in vitro* are injected into mice, either no infection results or the mice survive a very much longer time than do the controls. 4. Within certain limits, it is specific and can be used as an aid in the differentiation of trypanosomes. 5. The parasites which are refractory to their homologous serum, in other words, the parasites which exist in the blood simultaneously with the protective property, can be drawn from such blood and when subinoculated into mice remain resistant to that serum through many mouse passages (nineteen in one case). 6. The protective property is independent of any *in vitro* trypanocidal or agglutinative properties of serum. 7. When the trypanosomes are injected with the protective serum and examined in drops of peritoneal exudate at intervals of from a few minutes to one and a half hours, intense phagocytosis of the parasites can be observed. Taking into consideration the fixation of the property on the trypanosomes, the fact that the serum acts only when mixed with the virus, and the fact that within certain limits the longer the contact between the parasites and the immune serum before injection the greater the protective and phagocytic action in experimental mice, the authors suggested that the serums act by rendering the parasites phagocytable and that the protective property is similar to that of antibodies in general.

More recently, Heckenroth and Blanchard (1913 b), in repeating the work of Thiroux, found protective properties against *T. gam-*

biense in the serums of twenty-four out of twenty-five infected persons, whereas no such properties were found in the serums of five uninfected persons. In comparing this property with those of trypanolysis, leucocyte "attachment" and agglutination, they concluded that the serums of infected patients are almost always protective and frequently trypanolytic, that they rarely produce "attachment" and are only exceptionally agglutinative. The next year Ogawa (1914) described a protective power arising in the serums of guinea-pigs infected with *T. pecaudi* (= *T. brucei*).

The fact, brought out by Mesnil and Brimont, that the serum of an infected animal is protective against the strain of trypanosomes originally injected, but not against the particular relapse strain living in the animal, is clearly demonstrated by the protocols in Tables 17 and 18 giving some unpublished data of the author and Miss Robey. For these experiments, a sheep was infected with a strain of *T. equinum,* which was maintained throughout the experiment as

TABLE 17

PROTECTIVE EXPERIMENT WITH SERUM * OF A SHEEP INFECTED WITH T. EQUINUM AND TESTED IN MICE AGAINST THE PASSAGE STRAIN OF TRYPANOSOMES † (UNPUBLISHED DATA OF THE AUTHOR AND ROBEY)

Serum dose per 20 gm. mouse	*Weight of mouse*	*Dose of trypanosomes*	*Day on which trypanosomes appeared in blood*	*Day on which mouse died*
c.c.	gms.	c.c.		
2.0 undiluted	17.5	0.1	—	∞ ‡
1.1	19.0	0.1	—	∞
0.9	19.0	0.1	—	∞
0.7	19.0	0.1	—	20 §
0.5	21.0	0.1	—	∞
0.3	23.0	0.1	—	8 §
1.0 of 1:10	23.5	0.1	—	32 §
0.8	23.5	0.1	—	8 §
0.6	23.5	0.1	8	10
0.4	23.5	0.1	7	10
0.2	25.0	0.1	9	11
0 Control	28.5	0.1	4	6
0 Control	26.3	0.1	4	6

* This serum was infective when first isolated, but it was stored on ice until non-infective before use.

† The sheep was originally infected from a mouse strain which was maintained as a passage strain by being kept continuously in mice throughout the experiment.

‡ ∞ = Mouse discarded after being negative for 38 days.

§ Mouse died from intercurrent infections or by accidental means without showing trypanosomes in its blood.

TABLE 18

PROTECTIVE EXPERIMENT WITH SERUM * OF A SHEEP INFECTED WITH T. EQUINUM (SERUM OBTAINED AT THE SAME TIME AS THAT USED IN TABLE 17) AND TESTED IN MICE AGAINST A STRAIN OF ORGANISMS ISOLATED SIMULTANEOUSLY WITH THE SERUM † (UNPUBLISHED DATA OF THE AUTHOR AND ROBEY)

Serum dose per 20 gm. mouse	*Weight of mouse*	*Dose of trypanosomes*	*Day on which trypanosomes appeared in blood*	*Day on which mouse died*
c.c.	gms.	c.c.		
4.0 undiluted	17.0	0.1	3	6
3.0	17.0	0.1	3	7
2.0	17.0	0.1	4	10
1.1	17.0	0.1	1	5
0.9	18.0	0.1	1	7
0.7	18.0	0.1	3	6
0.5	18.0	0.1	1	5
0.3	18.0	0.1	1	5
1.0 of 1 : 10	18.5	0.1	1	7
0.8	19.0	0.1	1	5
0.6	19.0	0.1	3	5
0.4	19.5	0.1	3	5
0.2	20.0	0.1	3	5
0 Control	18.0	0.1	1	4
0 Control	18.0	0.1	3	5

* This serum was infective when first isolated, but it was stored on ice until non-infective before use.

† Between the time of isolation and the time of this experiment these trypanosomes were kept by passage in mice.

a passage strain by being continuously kept in mice. After some time the sheep was bled and the serum saved while the trypanosomes found at this time in its blood were injected and also continuously passed in mice until needed. Subsequently, when this serum was tested against the passage strain of trypanosomes (originally injected into the sheep) and the relapse strain (obtained at the time of bleeding), it completely protected mice injected with the passage strain in doses probably as low as 0.1 c.c. of undiluted serum (the exact minimal dose could not be ascertained because several mice died of intercurrent infections), whereas it did not protect mice injected with the relapse strain even in a dose as high as 4.0 c.c. per 20 gm. mouse.

The possible antibody basis for both the protective and curative actions of immune serum will be considered later (see page 156).

2. *Curative action of immune serum.* Most of the earlier workers were unable to demonstrate any curative property of immune serum

in trypanosome infections. Thus, Massaglia (1907) could obtain no prolongation of life in mice infected with *T. evansi* after injecting them with 1 to 2 c.c. of lytic serum (obtained from guinea-pigs during a trypanolytic crisis). Similarly, as I have already noted, many investigators found an immune serum highly protective but entirely without curative properties. Thus, Laveran and Mesnil (1912), writing of such protective serum, stated that "la dose de 1 et même 2 c.c. donneé chez la souris, n'influe en rien sur le cours de l'infection."

Positive results were, however, reported by two early investigators. Diesing (1905) reported that serum from donkeys after recovery and "hyperimmunization," with an undetermined species of trypanosome, when injected into infected cattle or horses, brought about a temporary disappearance of the parasites from the blood and a clinical improvement. Kleine and Möllers (1906) found that serum from donkeys after recovery from infections with *T. togolense* (= *T. brucei*) would bring about artificial crises in mice previously infected with the same species of trypanosome and showing one to two parasites per microscopic field. By repeated injections of the serum the life of these mice was prolonged five to thirty-four days over the control.

Mention should also be made here of the use of intraspinal injections of salvarsanized serum in sleeping sickness by Marshall (1920), supplementary details of which are given by Marshall (1921, 1921 b), Marshall and Vassallo (1921) and Eyre and Marshall (1921). The method consists of the following steps: (1) A patient is given an intravenous injection of neo-kharsivan, and after an hour or so, 10 to 40 c.c. of blood are withdrawn sterilely; (2) the blood is kept cool for twelve to sixteen hours so that the serum separates from the clot; (3) after a lumbar puncture, 15 to 20 c.c. of cerebrospinal fluid is removed and the same quantity of serum is slowly injected into the spinal canal. The authors believed that this method gave great promise in the treatment of the disease and suggested that the beneficial action of the serum was due to trypanolytic antibodies. These conclusions are open to question on a number of grounds which are summarized by Yorke (1921).

Van Saceghem (1922) used immune serum for the treatment of goats infected with *T. ruandæ* (= *T. congolense*). Five goats were infected from an ox, and six to ten days later, after parasites were visible in their blood, three goats were given 100 c.c. of serum from oxen with chronic infections; the injections were repeated weekly for five weeks with the result that the animals exhibited a low-grade infection and recovered in three months, whereas the two untreated

controls died. Animals thus cured were reported immune to subsequent reinfection (1922 b).

More recently, the present author and T. L. Johnson (1926) and T. L. Johnson (1929), working with *T. equinum* in mice, have shown that immune serums obtained from a variety of hosts (guinea-pigs and rabbits after the first crisis, mice after treatment with drugs and sheep during infection or after spontaneous recovery) are markedly curative in that they cause an apparently total disappearance of the parasites within a few hours and prolong the life of the treated animals over that of the controls. (For a description of an actual experiment, see page 123 and Figure 12.)

3. *Antibody basis for the protective and curative action of immune serum.* In evaluating this work, it seems to the author that the essential difference between the pathogenic and non-pathogenic trypanosome infections may be correlated with the fact that in the pathogenic trypanosome infections only parasiticidal antibodies are formed (lysins, opsonins), whereas in the non-pathogenic *T. lewisi* infections there is in addition the reproduction-inhibiting antibody (see page 125). For this reason the two types of infection had best be considered separately.

INFECTIONS WITH THE PATHOGENIC TRYPANOSOMES

In the work of the author and Johnson (1926) on the treatment of *T. equinum* infections with anti-*equinum* serum the curative action seemed to be simply a manifestation of lysis and to represent a repetition *in vivo* of what had frequently been observed *in vitro* because a study of slides showed the disintegration of the parasites in the blood-stream. Furthermore, the infections in mice practically always relapsed and terminated fatally, indicating that some of the parasites became resistant to the trypanolysins and repopulated the blood-stream, just as a few always do in infections progressing by crises and relapses.

In addition to the *in vivo* lysis by immune serum with its temporary curative effect, the work of the author and Johnson has demonstrated a number of peculiar facts in regard to the action of serum which probably account for the lack of positive findings of many previous workers and suggest a common basis for the protective and curative properties of serums.

According to the first paper (the author and Johnson, 1926), if various sized doses of immune serum be given to a series of mice in whose blood trypanosomes have just appeared, instead of lysis

TABLE 19

ZONE PHENOMENON IN THE IN VIVO TRYPANOLYTIC ACTION OF IMMUNE SERUM FROM A RABBIT INFECTED WITH T. EQUINUM (FROM THE AUTHOR AND T. L. JOHNSON, 1926)

Passage trypanosomes injected intraperitoneally into each mouse at beginning of experiment

Mouse number	Day of infection: 1	2	3: Number of parasites per field	3: Immune serum: intraperitoneally: Dose per 20 gm. mouse	3: Immune serum: intraperitoneally: Actual dose	3: Result in hours	4	5	6	7	8	9	10	11	12
	*			c.c.	c.c.										
1	+	+	20	2.5	2.9	−2	−	D							
2	+	+	10	2.3	2.9	+	+	D							
3	+	+	10	2.1	2.8	−2	−	+	+	+	+	+	D		
4	+	+	10	1.9	2.6	−2	−	−	−	+	+	+	+	D	
5	+	+	10	1.7	2.5	+	+	D							
6	+	+	10	1.5	2.2	+	+	D							
7	+	+	10	1.3	1.9	−2	−	−	−	+	+	+	D		
8	+	+	20	1.2	1.4	−2	−	−	−	+	+	+	+	D	
9	+	+	10	1.1	1.65	+	+	D							
10	+	+	10	0.9	1.35	−2	−	−	+	+	+	D			
11	+	+	10	0.7	1.08	−2	−	−	−	−	+	+	+	+	D
12	+	+	10	0.5	0.8	+	+	D							
13	+	+	10	0.3	0.5	+	+	D							
14	+	+	10	0.1	0.17	+	+	+	D						
15 Control	+	+	10	0	0	+	+	D							

* Plus sign indicates presence of trypanosomes in the blood; minus sign indicates their absence. No attempt is made to indicate the increase in parasites because once they appear in the blood they increase uniformly until death or until an artificial crisis is produced. During the relapse, after the artificial crisis, the same holds true.

being produced by doses greater than the minimal effective dose, there are recurring zones of lysis and non-lysis. This is obviously similar to the *in vitro* Neisser-Wechsberg phenomenon (page 28) and is well illustrated in Table 19. Here fifteen mice were all infected at

TABLE 20

RELATION BETWEEN NUMBERS OF TRYPANOSOMES IN TREATED MICE AND THE OCCURRENCE OF THE ZONE PHENOMENON (FROM T. L. JOHNSON, 1929)

Condensed protocols of seven series of experiments showing the length of life in days and occurrence of lysis in mice infected with *T. equinum* and subsequently injected intraperitoneally with doses of immune sheep serum when there were varying numbers of trypanosomes in their blood.

Series	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Approximate no. of parasites per field at time serum was given	1	5	10	15	20	28	50
Dose of serum per 20 gram mouse							
4.0 c.c.							7N
3.8							6N
3.6							6N
3.4							7N
3.2							7N
3.0							7N
2.9		7L*		5N		7N	5N
2.7	8L*	16L		18L	7L*	18L	6N
2.5	9L*	6L*	6L*	5N	7N	7N	6N
2.3	16L	7L*	19L	7N	18L	6L*	6N
2.1	16L	7L*	16L	6N	18L	7N	6N
1.9	16L	7L*	4L*	7N	6N	6N	6N
1.7	16L	16L	6N	18L	5N	6N	6N
1.5	16L	19L	9L*	6L*	18L	16L	6N
1.3	13L	16L	6N	8L*	8N	7N	6N
1.1	16L	19L	13L	11L*	7N	7N	6N
0.9	9L*	8L	15L	7N	6N	7N	
0.7	16L	19L	13L	6N		7N	
0.5	12L	15L				7N	
0 Control	7N	6N	6N	6N	7N	7N	6N

* Mouse died prematurely of causes other than trypanosomiasis.
L Serum caused lysis of trypanosomes.
N Serum did not cause lysis of trypanosomes.
The numbers before "L" and "N" represent the number of days each mouse lived.

the same time with the passage strain. When they showed from ten to twenty trypanosomes per standard field* (see third day of infection in the table), one was left untreated as a control, while the other fourteen were given various amounts of immune rabbit serum ranging from 0.1 to 2.5 c.c. per 20-gm. mouse. Within two hours, mouse 11, which received 0.7 c.c. of serum, showed a crisis, i.e., the trypanosomes disappeared from its blood. Mice 1, 3, 4, 7,

* Throughout this work a standard microscopic field represented a 4 mm. obj. and × 15 oc. Fresh preparations were examined.

TABLE 21

RELATION BETWEEN NUMBERS OF TRYPANOSOMES AND THE ZONE PHENOMENON FOR A SINGLE CELL STRAIN OF T. EQUINUM IN MICE TREATED WITH IMMUNE SERUM (FROM T. L. JOHNSON, 1929)

Number of trypanosomes per standard microscopic field at the time of treatment with immune serum	*Occurrence of the zone phenomenon*
1 to 5	No zone phenomenon; all doses greater than the minimal effective dose cause lysis
9 to 33	Zone phenomenon; doses greater than the minimal effective dose cause alternate zones of lysis and non-lysis
45 to 50	No lysis in any dose up to 4.0 c.c. per 20 gm. mouse

8, and 10 behaved in a like manner, whereas mice 2, 5, 6, and 9 did not have a crisis, did not show a diminution in the number of their trypanosomes, and died as soon as the control. (For many similar experiments see the author and Johnson, 1926.) Oddly enough, large doses of serum did not cause a mouse to live longer than smaller doses. No matter what the relative size of the doses of serum, when effective, they prolonged the life of the mice approximately equally, and when ineffective, the mice died virtually as soon as the control. In other words, the increase in length of life of the mice (i.e., the therapeutic value of the serum) was not dependent on the size of the serum dose, but simply on whether the serum dose produced lysis.

When this zone phenomenon was first discovered, it was thought to be characteristic of all infections, but later work by T. L. Johnson (1929) indicated that its occurrence was dependent on the number of trypanosomes in the mice at the time the immune serum was given, as shown in Table 20, which gives seven series of experiments. In series 1 and 2, where serum was given when there were one and five parasites per microscopic field, lysis occurred uniformly throughout. In series 3, 4, 5 and 6, where serum was given when there were about ten, fifteen, twenty and twenty-eight parasites per microscopic field, respectively, alternate zones of lysis and non-lysis occurred. In series 7, where serum was given when there were about fifty parasites per microscopic field, no lysis occurred in any

dose. These data are summarized in Table 21. The absolute number, moreover, varies with different single cell strains of trypanosomes. This characteristic in any strain is probably induced and of long duration, but is not a true genetical variation—it is, rather, similar to the acquisition of antibody resistance. Thus, Johnson was able to subject strain J4, which gave the zone phenomenon with ten to thirty parasites per field, to immune serum and secure a relapse strain which showed the zone phenomenon when there were one to five parasites per field. Parenthetically, it may be noted that these results, indicating the dependence of the zone phenomenon on the number of organisms in the mouse, effectively eliminate the criticism that the zone phenomenon may be simply an expression of the host's variability to the action of the immune serum. It is also of interest that Johnson (1929 b) has shown that neither normal human serum nor drugs ever elicit the zone phenomenon even if administered when enormous numbers of parasites are present in the mouse. As was indicated previously, these results have a certain bearing on previous work. The relation between the number of parasites and the activity of immune serum is an expression of common clinical experience in the treatment of certain diseases with immune serums. Thus, it has been found that good curative results are obtained if the serum is given very early in the disease, that variable results are obtained if it is administered later and practically no results if given after the malady is well established.

In this connection it is interesting to note that Coventry (1927) found that the zone phenomenon also occurred in protective experiments in mice with antipneumococcus serum and that its occurrence was dependent on the number of organisms injected simultaneously with the serum. The reader is referred to this paper for a discussion of relevant bacteriological literature.

In the first paper by the author and T. L. Johnson, it was suggested that the failure of many of the earlier workers to show lysis *in vivo* was due to the accidental use of doses where lysis was inhibited. In consideration of Johnson's later work, however, it seems more likely to have been due to the administration of serum when there were too many parasites in the test animal.

In view of Johnson's curative experiments in which the fewer the trypanosomes in the blood the greater and more uniform the action of the immune serum, and in view of the protective experiments reported by so many workers where comparatively few organisms were introduced and where the serum was not only uniformly effective but sometimes completely so, it seems to the present

author that the curative and protective properties of serums may be identical in their action and that the reason the protective property has seemed so much more certain is because with the fewer organisms used, the action of the serum is surer. In line with this suggestion, it is very simple to show that in protective experiments the use of too high an infecting dose (injecting too many parasites) will almost completely mask any protective action of serum. Thus, according to unpublished work by Johnson and the author, with a given amount of virus, doses of 0.1 c.c. of immune serum will completely protect, whereas with 1,000 times as many organisms, doses of immune serum as high as 3.0 c.c. per 20 gm. mouse may be without any marked effect (see Tables 22 and 23). If the two actions are identical, a lytic antibody is probably the basis of both manifestations. Provided that the destruction by lysis and by phagocytosis is fundamentally the same (see page 132), this contention would be in accord with Mesnil and Brimont's (1909) conclusions, that the protective property is manifest through the opsonizing effect of the immune serum and can be adsorbed by the bodies of the trypanosomes.

In concluding this section on the curative action of immune serums, it seems to me there has been little accomplished to justify the hope of producing serum definitely effective in the therapy of trypanosome infections. There are two inherent difficulties. In the first place, such serums are not effective unless used when there are few parasites. In the second place, even when the serums are effective by virtue of their lytic properties, some of the trypanosomes become resistant and repopulate the blood so that relapse almost invariably follows. These drawbacks have, of course, been overcome in the non-pathogenic *T. lewisi* infections by the combined presence of lysins and the reproduction-inhibiting antibody (see page 125), but so far only the lysins have been demonstrated in the pathogenic forms. It is true that an anti-reproducing antibody was postulated by van Saceghem (1923) for certain of the pathogenic forms, and although no immunological basis has been forthcoming as yet, it would, if it materialized, probably open up a new field for the development of curative serums.

INFECTIONS WITH T. LEWISI

Laveran and Mesnil (for a review, see 1912) obtained negative or very inconstant results in trying to treat (curative action) established infections with serum from rats that had recovered from an infection with *T. lewisi.* Coventry (unpublished work) has found that

TABLE 22

EXPERIMENT SHOWING PROTECTIVE PROPERTY OF SHEEP SERUM (AFTER RECOVERY FROM INFECTION WITH T. EQUINUM) AGAINST T. EQUINUM WHICH WAS OBTAINED FROM A HEAVILY INFECTED MOUSE WHOSE BLOOD WAS DILUTED 1:200,000 WITH SALINE (UNPUBLISHED DATA OF THE AUTHOR AND T. L. JOHNSON)

Mouse number†	Day of experiment*: 1 — Infected blood diluted 1:200,000	1 — Dose of serum per 20 gm. mouse	2	3	4	5	6	7	8	9	10	11	12
	c.c.	c.c.											
1	0.1	3.7	—	—	—	—	—	—	—	—	—	—	‡
2	0.1	3.5	—	—	—	—	—	—	—	—	—	—	‡
3	0.1	3.3	—	—	—	—	—	—	—	—	—	—	‡
4	0.1	3.1	—	—	—	—	—	—	—	—	—	—	‡
5	0.1	2.9	—	—	—	—	—	—	—	—	—	—	‡
6	0.1	2.7	—	—	—	—	—	—	—	—	—	—	‡
7	0.1	2.5	—	—	—	—	—	—	—	—	—	—	‡
8	0.1	2.3	—	—	—	—	—	—	—	—	—	—	‡
9	0.1	2.1	—	—	—	—	—	—	—	—	—	—	‡
10	0.1	1.9	—	—	—	—	—	—	—	—	—	—	‡
11	0.1	1.7	—	—	—	—	—	—	—	—	—	—	‡
12	0.1	1.5	—	—	—	—	—	—	—	—	—	—	‡
13	0.1	1.3	—	—	—	—	—	—	—	—	—	—	‡
14	0.1	1.1	—	—	—	—	—	—	—	—	—	—	‡
15	0.1	0.9	—	—	—	—	—	—	—	—	—	—	‡
16	0.1	0.7	—	—	—	—	—	—	—	—	—	—	‡
17	0.1	0.5	—	—	—	—	—	—	—	—	—	—	‡
18	0.1	0.3	—	—	—	—	—	—	—	—	—	—	‡
19	0.1	0.1	—	—	—	—	—	—	—	—	—	—	‡
20	0.1	0.05	—	—	—	—	—	—	—	—	—	—	‡
21	0.1	C	—	—	+	+	+	+	+	D			
22	0.1	C	—	—	—	+	+	+	+	+	+	+	D
23	0.1	C	—	—	+	+	+	D					
24	0.1	C	—	—	—	—	—	+	+	+	+	+	D

* Plus sign indicates presence of trypanosomes in the blood; minus sign indicates their absence; D indicates death of mouse; C indicates control.

† All mice weighed 16.4 grams.

‡ Mouse discarded 23 days later (35 days after beginning of experiment). No trypanosomes had appeared in its blood throughout that period.

§ Mouse died of other causes 21 days after beginning of experiment; no trypanosomes had appeared in its blood.

TABLE 23

EXPERIMENT SHOWING PROTECTIVE PROPERTY OF SHEEP SERUM (AFTER RECOVERY FROM INFECTION WITH T. EQUINUM) AGAINST T. EQUINUM WHICH WAS OBTAINED FROM THE SAME HEAVILY INFECTED MOUSE AS IN TABLE 22, WHOSE BLOOD WAS DILUTED 1:2 WITH SALINE (UNPUBLISHED DATA OF THE AUTHOR AND T. L. JOHNSON)

	Day of experiment *																
	1		*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
Mouse number†	*Infected blood diluted 1:2*	*Dose of serum per 20 gm. mouse*																
	c.c.	c.c.																
25	0.1	3.7	−	−	−	−	−	−	−	+	+	D						
26	0.1	3.5	−	−	−	−	−	−	−	+	+	+	D					
27	0.1	3.3	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	D
28	0.1	3.1	−	−	−	−	−	−	−	−	+	+	+	D				
29	0.1	2.9	−	−	−	−	−	−	−	−	+	+	+	D				
30	0.1	2.7	−	−	−	−	−	−	+	+	+	+	D					
31	0.1	2.5	−	−	D													
32	0.1	2.3	−	−	−	−	−	−	−	−	+	+	+	D				
33	0.1	2.1	−	−	−	−	−	−	−	−	+	+	+	D				
34	0.1	1.9	−	−	−	−	−	−	−	−	−	+	+	+	+	D		
35	0.1	1.7	−	−	−	−	−	−	−	+	+	+	D					
36	0.1	1.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+‡
37	0.1	1.3	−	−	−	−	−	−	D									
38	0.1	1.1	−	−	−	−	D											
39	0.1	0.9	−	−	−	−	−	−	−	−	−	+	+	+	D			
40	0.1	0.7	−	−	−	−	−	−	−	+	+	+	+	D				
41	0.1	0.5	−	−	−	−	−	−	−	+	+	+	D					
42	0.1	0.3	−	−	−	−	−	+	+	+	D							
43	0.1	0.1	+	+	+	+	+	+	D									
44	0.1	0.05	+	+	D													
45	0.1	C	+	+	D													
46	0.1	C	+	+	D													
47	0.1	C	+	+	D													
48	C	3.0	−	−	−	−	−	−	−	−	−	−	−	−	−	§		
49	C	1.0	−	−	−	−	−	−	−	−	−	−	−	−	−	§		
50	C	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	§		

* Plus sign indicates presence of trypanosomes in the blood; minus sign indicates their absence; D indicates death of mouse; C indicates control.

† All mice weighed 16.3 grams.

‡ Mouse died 3 days later of trypanosomiasis.

§ Mouse discarded with no trypanosomes in its blood (serum control for both series).

a trypanolytic crisis can be produced in such rats with an established infection, provided the number of trypanosomes is small. Furthermore, her work indicated that in a series of infected rats, if each rat was treated with a different dose of serum, the efficiency of the serum—i.e., whether lysis throughout the series, the zone phenomenon, or no lysis whatever occurred—depended on the number of parasites in the blood, as she (1927) had previously found in pneumococcus infections and Johnson (1929) had reported for *T. equinum*. These results not only explain the negative findings of Laveran and Mesnil, but indicate that the curative action of immune serum in the case of *T. lewisi* has the same parasiticidal basis as in infections with the pathogenic trypanosomes.

When the serum is introduced simultaneously with the virus, or nearly so (protective action), another possibility arises. Such experiments are similar to those by the author (page 125) in which the reproduction-inhibiting antibody was demonstrated, except that fewer organisms are used. Consequently the protective action in *T. lewisi* infections can be explained by the combined presence of the reproduction-inhibiting antibody and the parasiticidal antibody, whereby the former prevents cell-division of the parasites and the latter kills them. Parenthetically, it may be noted that the reproduction-inhibiting antibody probably plays no part in the curative experiments because all evidence indicates that it is not parasiticidal.

4. *Protective and curative action of normal serum.* A number of normal serums, particularly of primates, have been found to exert protective and curative actions *in vivo* against certain species of trypanosomes.* At first these might seem to be examples of natural antibodies, but the only case that has been carefully studied, i.e., normal human serum, has been found to be quite different from immune serums in its nature and action. For this reason attention will be limited to some of the papers establishing this difference, and, for the remainder of the work on this subject, the reader is referred to the admirable review by Laveran and Mesnil (1912).

The curative property of normal human serum was discovered by Laveran (1902) and both the curative and protective properties were studied by him and Mesnil. (See Laveran and Mesnil, 1902; Laveran, 1903 and 1904; and Mesnil, 1915.) Thus, a dose of 0.1 to 1.0 c.c. caused the disappearance of the trypanosomes from the circulating

* Although the present discussion is limited to the action of normal serums on the pathogenic trypanosomes, many other cases could be cited. For example, Thiroux (1909) found that normal sheep serum was protective against infections with *T. duttoni* in the mouse.

blood of a mouse infected with nagana in twenty-four to thirty-six hours. Individual serums varied extensively and that of adults was much more effective than that of infants. The authors did most of their work with *T. brucei* in rats and mice, but found a similar action against *T. evansi, T. equinum* and to a less extent against *T. dimorphon* (= *T. congolense?*).

Goebel (1907) checked the early work of Laveran and Mesnil and amplified it with the following findings: The protective activity of normal human serum is gradually decreased with increasing temperature until it is finally destroyed at 64° C.; the protective property is not fixed on the trypanosomes *in vitro* for it is retained by serum which has been in contact with trypanosomes at 37° C. and subsequently tested after their removal, while the parasites are still infective; the protective property is carried down with the globulins on saturation with magnesium sulphate; and furthermore, it is lost (the curative activity is only diminished) when human serum is mixed with rabbit anti-human serum. From his experiments it is impossible to say that this last characteristic is due to the formation of an anti-protective substance in the antiserum, because if any precipitating mixture (such as horse serum and rabbit anti-horse serum or even normal serums of various animals) be added to the human serum, the activity is more or less diminished. Finally, *in vitro* experiments failed to give any evidence that the human serum acted by rendering the parasites phagocytable. In contrast to these attributes of the protective property of normal human serum it will be recalled that the protective property of immune serum, as demonstrated by Mesnil and Brimont (1909), resists heating to 64° C. and is fixed on the bodies of the parasites so that it is possible to sensitize them *in vitro*. Thus, the two phenomena seem basically different. This has been established beyond doubt by Rosenthal and his co-workers in their very fruitful investigations.

Rosenthal and Kleemann (1915) verified and extended the earlier observation of Laveran and Mesnil that the trypanocidal property was weaker in fetal serum collected at birth than in adult serum, as is shown by one of the protocols of Rosenthal and Kleemann given in Table 24. Such results were obtained both protectively and curatively. Furthermore, it seemed appreciably increased in the mother's serum during the last period of gestation, and, according to the previous work of Neumann (1911), persisted so for some time after delivery. These results indicated that the substances responsible for the trypanocidal property do not pass the placenta and are not formed in the prenatal stages of development. Many of these conclusions are

TABLE 24

PROTECTIVE ACTION OF NORMAL SERUM FROM MOTHER AND FETAL BLOOD AGAINST T. BRUCEI (SLIGHTLY MODIFIED FROM ROSENTHAL AND KLEEMANN, 1915)

Serum introduced subcutaneously and trypanosomes intraperitoneally at beginning of the experiment.

Mouse number	*Serum dose in c.c.*		*Days after infection*													
			1	*2*	*3*	*4*	*5*	*7*	*9*	*11*	*13*	*15*	*16*	*18*	*22*	*26*
1	1.0	Mother's serum	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	0.5	Mother's serum	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	0.5	Mother's serum	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	0.25	Mother's serum	−	−	−	−	−	−	−	−	−	−	+	+D		
5	0.25	Mother's serum	−	−	−	−	−	−	−	−	−	−	+	+D		
8	1.0	Child's serum	+	+	+	+	D									
9	0.5	Child's serum	−	+	+	+	+D									
10	0.5	Child's serum	+	+	+	+	D									
11	0.25	Child's serum	+	+	+	+	D									
12	0.25	Child's serum	+	+	+	D										
6C	0		+	+	D											
7C	0		+	+	D											

D = Death of mouse.
− = No trypanosomes in the blood.
+ = Trypanosomes in blood (although not shown in the present table, the original results of Rosenthal and Kleemann indicate that once these appeared in the blood they increased at a uniform rate until death of the mouse).
C = Control.

further supported by the work of Neumark and Pogorchelsky (1925) and Nattan-Larrier and Lépine (1927). Neumark and Pogorchelsky presented evidence to show that the property did not appear until the eleventh to fifteenth week, but S. Levy (1928) found it already present by the third week.

Another interesting aspect of the problem was forthwith attacked. Rosenthal and Krueger (1921) studied the decrease of trypanocidal properties in the serums of persons suffering from certain diseases of the liver which had been previously noticed by Ehrlich (1908). (See also Platau, 1916; Zeiss, 1921; and Saito, 1927.) Quite in line with other workers, Rosenthal and Krueger found that a protective dose of 0.1 c.c. of normal serum per 20 gm. mouse never failed to be definitely trypanocidal in mice infected with *T. brucei*, whereas the property was greatly diminished in patients suffering from mechanical jaundice or severe diffuse liver diseases. In studying the mechanism of the decrease they found that the condition occurred in severe liver disease without icterus, but only in icterus accompanying dis-

eases of the liver. Furthermore, it was definitely reduced when human bile was added to normal serum in the proportion of one to three, but not ordinarily when bile was added in the proportion of the bilirubin concentration of icteric serum. From these experiments they concluded that the trypanocidal property stands in close relationship to functional activities of the liver and is not diminished primarily on account of the toxicity of the bile constituents. Further evidence in support of these conclusions was supplied by Rosenthal and Nossen (1921) and Rosenthal and Freund (1922 and 1923). In the last mentioned paper, the following supplementary conclusions were drawn. The protective substances are associated with the euglobulin and pseudoglobulin fraction of the serum and not with the fibrinoglobulin and albumen fraction. The property is not antigenic because specific antibodies could not be induced in rabbits and mice by immunization with active normal human serum. Large and repeated doses of active human serum inhibit the activity of a subsequent dose. This process is not, however, an immunization but represents an exhausting of some system within the treated animal which normally is stimulated by the human serum to produce trypanocidal substances. From this it follows that the human serum does not contain trypanocidal substances *per se* but rather trypanocidogenic substances which stimulate the body of the animal to produce the actual trypanocidal substances. Rosenthal and Spitzer (1924) later added the evidence that this was accomplished through the mediation of the reticuloendothelial system in mice. (For details of this work see page 240.)

In the past, many investigators assumed that since human serum exerted a protective and curative action on the pathogenic trypanosomes of animals, which do not infect man, but was ineffective against *T. gambiense,* which does infect man, the trypanocidal property of human serum was the basis of man's natural immunity. The trend of the work just reviewed, however, clearly points to the indirect nature of the protective and curative action of normal human serum and strongly indicates the extreme unlikelihood that this phenomenon has any bearing on the natural immunity of man against the animal trypanosomiases. Direct corroborative evidence is available in the experiments with trypanosomes which have become resistant to the action of human serum. Thus, Jacoby (1909) obtained a strain of *T. brucei* resistant to a dose of 2 c.c. of human serum. The question arises, Can these infect man? Two pieces of work indicate that they cannot. Mesnil and Leboeuf (1912) obtained a strain of *T. gambiense* resistant to baboon serum which upon injection into a young baboon

did not produce an infection. Objection has been raised to this work because the baboon, unlike man, is resistant to practically all trypanosomes. Therefore, Collier (1924) obtained a strain of *T. brucei* which was resistant to human serum and injected himself intramuscularly with 200 million trypanosomes, but remained uninfected. Four other persons, three injected subcutaneously and one intravenously, also gave negative results. Rosenthal and his co-workers' conclusions are thus borne out by experimental evidence.

Recently, considerable interest has been aroused in the trypanocidal properties possessed by serums from persons suffering with various diseases. Jaffé and S. Brown (1927), in a study of five patients undergoing malaria treatment for paresis, found that the trypanocidal property of such serums diminished during malaria chills, reached a minimum just after the temperature returned to normal, but reappeared in full strength before the next chill. Likewise, the property was greatly diminished in certain cases of hæmophilia during various stages of the disease and might be absent in their non-bleeder relatives. (See Leichtentritt and Opitz, 1927, and Leder, 1928.) On the other hand, the serum from persons suffering with Hodgkin's disease (Zeiss, 1921 b) was no less effective than normal serum in its action on *T. brucei* and only slightly less so on *T. rhodesiense*. Leichtentritt and Zielaskowski (1922), in comparing the trypanocidal property of the serums of healthy children with those suffering from Barlow's disease, found a decrease of trypanocidal power during the acute stage, with an increase as convalescence commenced. Continuing this work Grünmandel and Leichtentritt (1924) stated it as their conviction that the trypanocidal property is diminished in conditions dependent upon a lack of vitamins. Thus, a complete absence of the property was found in children suffering from corneal ulcers associated with a lack of high-grade fats and poor milk, and in children suffering from alimentary edema associated with an improper diet and complicated by secondary infections.

2. PIROPLASMOSIS

Just as in trypanosome infections, the serums of dogs infected with *Babesia canis* acquire the power to protect other dogs against infection with the virus. Thus, according to Nocard and Motas (1902), the blood of a dog, apparently cured, exerted a protective action when injected with the virus. Theiler (1904) confirmed this, and also found that in cases apparently cured, a labile infection existed. Here again, then, the protective property coexists with the parasite.

3. MALARIA

The only positive results with immune serum in malaria, of which the author is aware, are two reports on the human disease. In the work of Sotiriadès (1917) the immune serum was obtained from an Athenian whose blood for many months had shown crescents, which treatment did not affect, although it improved clinical symptoms. Ten c.c. of this serum was administered subcutaneously into a second patient with ring forms in his blood. enlarged spleen, fever, nervous disturbances, and profound debility. Within eight hours, symptoms had disappeared and his temperature had fallen from 103.6° F. to 99.8° F. Four days later, a relapse indicated by a milder paroxysm responded within four hours to a second dose. A third dose was given the next day and the patient discharged twelve days later, having been free of fever during the interval.

Working on induced malaria in paresis, Kauders (1927) noted the fact that a second infection was not only difficult to effect, but when produced, required repeated injections of the virus and resulted in an infection with a prolonged incubation period which died out in four or five attacks. One or two injections of 1 to 2 c.c. doses of serum from such patients after recovery proved of benefit to nine out of twelve infected patients and five of the nine recovered from their fever, which could not be provoked and did not return spontaneously, whereas similar injections of serum from normal persons or those suffering from acute malarial attacks were ineffective. In studying the protective property of the immune serum, it was found that seven to nine injections of serum before the introduction of the virus did not protect, but rather shortened the incubation period; whereas eight injections of serum after the introduction of the virus gave the following results: one patient did not acquire malaria in spite of twelve subsequent injections and a number of provocative treatments, two had only four or five attacks and three showed the usual course of the infection.

Although caution must be observed in drawing too close an analogy between the avian and human infection, because complement-fixing and precipitin antibodies can be demonstrated in the human but not in the avian disease, it is interesting to note that neither protective nor curative properties of serum taken at various stages in infections with bird malaria could be demonstrated by the author and L. G. Taliaferro (1929 b).

II. *Helminth Infections*

As a result of his experimental work, Salzer (1916) maintained that the serum of patients recovered from trichinosis exerted a curative and prophylactic action against infections with *Trichinella spiralis*. He injected animals with immune serum either simultaneously or within twenty-four hours of their being fed trichinous meat and found that they did not develop trichinosis; whereas control animals fed only trichinous meat invariably developed the disease. Furthermore, in two human patients, the administration of immune serum during the active stage of the disease exerted a remarkable curative action. Schwartz (1917), as a result of animal experimentation, disagreed with Salzer's conclusions. His experiments indicated that serum from animals convalescent from trichinosis did not produce any immunity to trichinosis when injected into non-infected animals prior to feeding trichinous meat; that it exerted no prophylactic action when fed together with the infected meat; that it exerted no ill effects *in vitro* on trichina larvæ which had been freed from their cysts; and furthermore, he failed to observe any immunity to further infection in animals that were once infected and still harboring trichinæ.

Hall and Wigdor (1918) attempted to differentiate the effect of the convalescent serum on the trichinæ themselves and on the toxic action of the trichinæ. Working with rats they concluded, in agreement with Schwartz, that convalescent serum does not inhibit the development of trichinæ. On the other hand, in agreement with Salzer, they concluded that such a serum may be of decided value in combating the toxic features of trichinosis. The data, nevertheless, seem insufficient as yet to warrant such a conclusion, since Salzer made observations on only two cases and Hall and Wigdor found that only nine out of fifteen rats lived longer than the controls. In two human patients Alexander (1923) obtained no appreciable effect on temperature, eosinophile count, or duration of the disease by the use of four 10 c.c. injections of convalescent serum.

In conclusion, the work done so far indicates very strongly that convalescent serum from cases of trichinosis does not produce any immunity or exert any parasiticidal or inhibitory action on the worms themselves. As to whether or not it may exert a curative or prophylactic action on the toxic effects of the worms, the data are entirely inconclusive.

III. *Summary*

The serums of a large number of animals, infected with trypanosomes and after spontaneous recovery and after drug therapy, or during the subacute or chronic course of infection, may possess a protective property against the passage strain of specific trypanosomes. If the immune serums are obtained during infection, they are ineffective against the strain of the parasite isolated from the animals simultaneously with the immune serum. The protective property is tested by injecting the immune serum approximately simultaneously with the specific organism into an experimental animal, generally a mouse, and is manifested either by a complete inhibition of the infection or by a longer survival time of the experimental animal as compared with a control given only the organism. In the pathogenic trypanosomes, the protective property is specific within certain limits; can sensitize the organisms *in vitro;* and is probably dependent upon parasiticidal antibodies (lysins, opsonins). In the non-pathogenic *T. lewisi* infections the protective property is probably dependent not only upon the parasiticidal antibodies, but also upon a reproduction-inhibiting antibody.

Immune serums which are protective may be highly effective curatively if injected into animals (generally mice) with a fairly well-established infection. The curative activity of the immune serums, against both the pathogenic and non-pathogenic trypanosomes, if administered to a series of animals in graded doses, exhibits a peculiar relation to the number of parasites present at the time the serum is given. When there are very few parasites, the serum lyses the parasites in the whole series (above the minimal effective dose); when there are more parasites present (the exact number differs for each strain), the serum shows recurring zones of lysis and non-lysis (above the minimal effective dose); when the parasites are numerous, no lysis whatever occurs.

Serums of dogs infected with *Babesia canis* acquire a protective activity, and serums from man infected with malaria may possess a curative activity.

Normal serum also may exert a protective and curative action against certain trypanosomes. Experiments with normal human serum, tested against *T. brucei,* seem to offer conclusive evidence that normal serum does not contain trypanocidal substances *per se* but rather trypanocidogenic substances which act only through the mediation of the reticulo-endothelial system of the experimental ani-

mal. The curative activity of normal serum is not dependent on the number of parasites present at the time it is administered, but consistently lyses the parasites (above the minimal effective dose) in a series of mice. Drugs seem to behave in a manner similar to normal serums. The trypanocidal property of human serum is absent in newborn infants and may be greatly decreased in certain diseases, especially those involving organic lesions of the liver.

CHAPTER V

HYPERSENSITIVENESS AND VARIOUS CUTANEOUS TESTS USED TO DETECT PARASITIC INFECTIONS

I. *Introduction*

No field of parasitic immunity offers more promise of interesting practical and theoretical results than does the study of various skin reactions associated with infection. As the reactions considered in this chapter are expressions of more general changes in the body, it is to be noted that if the inciting substance is introduced into other locations, as for example the conjunctiva, other types of reaction ensue. Since few authors so far have considered this field as a whole, it seems desirable at the outset to classify the more important types of skin responses encountered in immunological work. Such a classification is, of necessity, tentative, because the mechanisms and relationships involved are still very obscure. Moreover, no attempt will be made to classify the ophthalmic tests, because the available data on their nature are as yet too meager.

The intracutaneous tests fall naturally into two categories: those like the tuberculin test and hay-fever tests, which depend upon hypersensitiveness to certain materials, and those like the Schick test, which are dependent upon injury to normal tissues by a definite toxin in the absence of the specific antibody. Especial attention will be paid to the skin tests dependent upon hypersensitiveness, all of which involve the introduction of a small amount of the test material into the skin. The test material consists of powdered parasites, various suspensions of such parasites, or extracts of them, and may be applied to abrasions in the skin or, in the case of suspensions or extracts, may be injected intracutaneously. Where the injection method is used, the amounts vary, but rarely exceed 0.1 c.c. A control test with the diluent or extractive (and in some cases with other proteins) is necessary to eliminate certain types of pseudo-positives and is carried out at the same time as the specific test. Variations in technique will be brought out in the subsequent descriptions.

I. HYPERSENSITIVENESS

Hypersensitiveness, that is, a greater sensitiveness than is considered normal, is exhibited by individuals for a number of materials such as pollens, animal dander, serums, plant poisons and drugs. Doerr has used the term "allergy," first introduced by von Pirquet, to signify all cases of altered reactivity (either increased or decreased) whether to antigenic substances or to non-antigenic materials. Some authors have definitely limited the term allergy to hypersensitivity to antigenic substances, others have variously defined and classified it, and still others have discarded it altogether because of a lack of general agreement of what it should signify.

There are so many variations in the exact manifestation of the different types of hypersensitiveness that it is impossible to decide at the present time into how many categories they fall. One prominent authority in the field (Coca, 1925) defines hypersensitiveness as "a sensitiveness that is mediated by a special mechanism, which may be influenced by suitable exposure to the exciting agent in the direction of lessened or increased sensitiveness." He then classifies the various forms as follows:

Hypersensitiveness

Normal	Abnormal
a. Serum disease (ordinary)	a. Anaphylaxis
b. Dermatitis venenata	b. Hypersensitiveness of the tuberculin type
	c. Atopy

He considers that the two diseases placed under "normal" may affect the majority of individuals of the species, whereas the three conditions under "abnormal" may affect individuals only if they happen to possess the proper previous exposure, and in the case of atopy, the proper inherited complex.

In any case, for the sake of clearness, it is essential to outline at least three types of skin reactions which are of the hypersensitive type and which in Coca's classification fall under the three types of abnormal hypersensitiveness.

In dealing with these it is impossible to apply the results from one animal species to another. Each case has to be worked out separately because the reactions of the "shock organs" vary in different animals. Moreover, it is quite possible to obtain different reactions

in the same test. For example, the injection of tuberculin intradermally into a tuberculous person may be followed first by an immediate reaction and later by a typical tuberculin reaction.

1. *Immediate skin reactions; protein wheals; atopic hypersensitiveness.* Under these terms are included certain conditions, notably hay-fever and asthma, in which the intradermal introduction of a small amount of an extract of the exciting agent is followed in hypersensitive individuals by the formation of a wheal of varying size, with irregular margins (pseudopods), surrounded by a zone of erythema. The reaction is very quick compared to the other types of cutaneous tests and reaches its maximum in from ten to twenty minutes. (See Figure 22.)

This type of hypersensitiveness can be demonstrated cutaneously by three methods, (a) rubbing the powdered exciting agent into scratches on the skin, (b) placing an extract of the material on the skin and then making superficial scratches, or (c) injecting intradermally 0.01 to 0.1 c.c. of aqueous extracts (most efficient, and allows quantitative analysis). It can also be demonstrated by the ophthalmic test where the material may be instilled into the conjunctival sac, causing congestion, itching, lacrymation and sneezing. (These clinical manifestations may be controlled by instilling a drop of 1 : 1,000 adrenalin chloride into the sac.) In this test the severity of the reaction is dependent upon the concentration and not the quantity of the exciting agent. Since individuals occasionally give a pseudo-positive reaction toward any of the numerous diluents used in extracting the test material, if a diluent is used, a control test with it alone always accompanies any test.

Among the characteristics of this type of cutaneous reaction, the following are noteworthy: The sensitivity is passively transferable to a non-sensitive individual. Thus, as Prausnitz and Küstner (1921) first showed, the serum of a sensitive person will locally sensitize a normal individual for a few days if injected into the skin. The sensitizing property differs from an anaphylactic antibody in that it will not passively sensitize a guinea-pig's uterus and will not render the specific exciting substance inactive when the two are mixed *in vitro*. Also, desensitization, in the sense used in connection with anaphylaxis, does not occur. Thus, in most persons who are clinically "desensitized" by repeated injections of the inciting agent, there is a retention or even an augmentation of the skin reaction. Rapidly repeated injections of the inciting agent into the same locality will seemingly desensitize, but this is a local exhaustion of the physiological mechanism and is not specific and does not exhibit the specific

desensitization found in anaphylaxis after a single sublethal injection. (See Coca, 1928.) Recently Bloch and Karrer (1927) have isolated a crystalline glucoside from the primrose that will incite the reaction in hypersensitive persons. (Cf. the work of Avery and Heidelberger on soluble specific substances, page 9.) Coca considers the serum property responsible for passive transfer so different from a true antibody that he classifies it with the serum elements responsible for the Wassermann reaction as a reagin and specifically refers to it as an atopin. The condition and the age at which the sensitivity appears can apparently be developed only in those individuals who have inherited a definite predisposing complex. Coca, however, believes that in the case of the intestinal helminths it can be developed by all persons with suitable exposure—in other words, is not inherited in the sense that it is in hay-fever and asthma. This view will be discussed later (page 193). Although Coca (1928) thinks it very unlikely that this type of hypersensitivity can be developed in the lower animals, other workers have observed a closely similar, if not identical, reaction in guinea-pigs. The exact relationship of the immediate reaction in man and guinea-pigs is unknown.

2. *Delayed or tuberculin type of reaction: hypersensitiveness of infection* (*Coca*). This type of reaction is exhibited in various bacterial and parasitic infections. The nature of the reaction can best be described by reference to the best-known example of the type, the tuberculin reaction. The injection of tuberculin into the skin of a normal animal is followed by a few transitory microscopic changes at the site of injection, which disappear within a few hours. The same material when injected into the skin of a tuberculous person is not followed by any immediate reaction (unless the individual is also atopically hypersensitive) as in the hay-fever type of reaction; but after from eight to twenty-four hours, an intense local inflammation occurs at the site of the injection. "A red, raised area, with central blanching, 1-2 cm. in diameter, appears, which is of firmer consistency than the surrounding skin. If the animal is exceptionally sensitive, or the tuberculin solution concentrated in active principle, central necrosis occurs. Microscopically, the capillaries are seen to be dilated and engorged, and an intense infiltration of large mononuclear and small polynuclear leukocytes is observed. There is an increase in the intercellular fluid, and some coagulated fibrin may be present" (Long, 1928). The reaction is highly specific, but Long (1928) feels that in the last analysis its specificity will be found, as in other immunological reactions, to lie in its quantitative aspect. This view is strongly supported by the group reactions noted in Chapter IX.

As in the previous test this reaction may be carried out by the skin-scratch or skin-injection method or by instillation into the ocular conjunctiva. It may also be carried out by injection into such parenchymatous organs as the testis. (For a description of the last method see Long, 1928.)

The immunological characteristics of this reaction, as well as its differences from the last, can now be outlined. No inherited complex is necessary as most individuals can be sensitized by infection. At first, in fact, infection was supposed to be a necessary prerequisite, but some investigators have found that large amounts of dead tubercle bacilli will sensitize animals to tuberculin. Similarly, animals immunized with extracts of parasites may exhibit what appears to be the same reaction. (Cf. Wagener, 1923; Bachman, 1928; and Coventry, 1929.) In a sensitive animal, the reaction may be elicited by extracts of numerous infective agents; and according to the extremely valuable studies of Seibert (1928) the tuberculin reaction is elicited by tuberculin which she has isolated and crystallized in the form of a water-soluble protein which is a true antigen (precipitinogen).

Unlike the preceding and succeeding phenomena, this sensitivity cannot be transferred passively to the skin of another animal. It is true that Zinsser has been able to sensitize the uterus of guinea-pigs by repeated injections of serums from tuberculin-sensitive animals, but this is not a transfer to the skin and may be a concomitant anaphylactic phenomenon rather than the true tuberculin sensitivity. The absence of a sensitizing property in the serums of sensitive animals would seem to differentiate this reaction from local anaphylaxis, but it may be a similar mechanism in which the antibodies are limited to the cells. The reaction is elicited in various infections (and after immunizing procedures) in man and a large number of animals.

3. *Local anaphylaxis: the Arthus phenomenon.* This type of skin reaction follows the intradermal injection of a protein into a rabbit anaphylactically sensitive to that protein, and seems to be one of local anaphylaxis, i.e., the antigen and antibody react locally at the site of injection. Clinically, it involves acute inflammation and possible necrosis which take time to develop, and thereby superficially resembles the hypersensitiveness of infection reaction. The sensitivity can be passively transferred with the serum of a sensitive animal. According to Opie (1924, 1924 b and 1924 c) and Opie and Furth (1926), passive transfer can be reversed. Thus, rabbits were injected intravenously with a protein (antigen) and within several hours were injected intracutaneously with an appropriate antiserum, with the

result that a reaction similar to the Arthus phenomenon occurred. In accordance with its anaphylactic nature, serum from a rabbit in the sensitized condition will passively sensitize a guinea-pig's uterus, and will render the specific exciting substance inactive upon contact *in vitro*.

Local anaphylaxis occurs in the skin of rabbits and in certain organs of the dog. It may also occur in the skin of guinea-pigs, although the results so far are conflicting.

2. TOXIN-ANTITOXIN REACTIONS IN THE SKIN

The outstanding practical use of this type of skin reaction is the Schick test for antitoxic immunity in diphtheria. The test is carried out by the intracutaneous injection of one fiftieth of the minimal lethal dose for a 250-gm. guinea-pig (M. L. D.) of diphtheria toxin. In an individual having no antitoxic immunity (i.e., a susceptible person), a reaction develops twenty-four to forty-eight hours thereafter, consisting of an area of erythema, swelling and sometimes superficial necrosis; whereas, in immune individuals, no reaction occurs. Some individuals are susceptible to the proteins, other than the toxin, in the material, so that a control test is made with detoxicated material (toxin heated to 75° C. for ten minutes). This test differs from those depending upon hypersensitiveness in that a positive reaction occurs in the normal individual.

II. *Amœbiasis*

The results of Scalas (1923) indicate that an intradermal reaction may be possible as a practical test for amœbic dysentery in man. The test material was prepared by mixing 30 grams of mucus and fragments of intestinal mucosa obtained from the fresh feces of an acute case of amœbic dysentery with 30 c.c. of physiological saline in a flask with a few glass beads, incubating for a week at 37° C. with daily shaking, filtering first through paper and then through a Berkefeld candle, decolorizing with animal charcoal and sterilizing. In positive reactions the injection of 0.25 c.c. intradermally was followed in about one hour by swelling and erythema accompanied by itching and a sense of heat but no pain. These signs all disappeared in from one to three days. In negative reactions there was only an erythema. Tests on thirty-two persons gave the following results: positive reactions were obtained in nine cases of acute, subacute, or chronic amœbic dysentery, and negative reactions in twenty-three persons, of

whom five were healthy, seven had non-amœbic entero-colitis, and eleven had various other non-amœbic diseases. It would be very interesting to repeat these experiments of Scalas, using a test material containing a higher concentration of amœbic substances, possibly obtained from cultures, such as Craig used in his study of complement fixation in amœbiasis (page 32).

III. *Leishmaniosis*

The interest in intradermal tests in the *Leishmania* infections has centered around the delayed type of reaction. Although occasional incidental references have appeared on reactions in infected humans when leishmanial products were injected, credit is due Wagener (1923) for the first careful study of the intradermal test. The material she used for intradermal injection was prepared from both *L. tropica* and *L. donovani* (*infantum* strain) as follows: cultures were washed in saline, centrifuged, the sedimented organisms diluted with Coca's solution (NaCl 0.5 per cent, $NaHCO_3$ 0.05 per cent, and phenol 0.4 per cent) until there were 2,000,000 organisms per c.c., covered with toluol and allowed to stand at room temperature for three days. After this period the suspensions were centrifuged and the supernatant decanted into sterile tubes ready for use. Four rabbits, two of which had been immunized with each of the two strains of *Leishmania,* and one normal rabbit were tested by injecting 0.2 c.c. of the suspension (actual test) and 0.2 c.c. of the diluent (control test) into two prepared sites on the skin. The immunized animals showed, at the site of the actual test, a small reddened papule at the end of twenty-four hours, which reached its height at forty-eight hours and persisted for five days, whereas neither the tests on the normal rabbit nor the control tests on the immunized rabbits showed any reaction. This is obviously a delayed type of reaction and is identified by the author as probably Coca's "hypersensitiveness of infection." Tests with materials prepared from proteins of the flagellates which had been precipitated with alcohol were all negative. There was no indication of specificity in the reactions between the two strains of *Leishmania.*

Using essentially Wagener's technique, Montenegro (1926) studied the practicality of using the test in diagnosing the Brazilian type of cutaneous disease. His description of the resulting reaction is typical of the delayed type. In thirty-seven infected patients thirty-two gave a positive test; of thirty-six uninfected controls thirty-three were negative and three doubtful. A test fluid made with *L. tropica*

reacted with the Brazilian cases, but this is not surprising in view of Wagener's (1923) and Wagener and Koch's (1926) demonstration of the lack of species-specificity in this test.

Working with *L. tropica,* Jessner and Amster (1925) reported an intradermal reaction of forty-eight hours' duration in three infected dogs and in one person infected with kala-azar. In five healthy dogs and one recovered dog the reaction was negative.

I know of no work on the occurrence of the immediate wheal type of reaction in humans infected with *Leishmania.* In conjunction with Dr. Frances Coventry, I carried out a few such tests in Tela, Honduras, using the scratch method and a test material from *L. tropica* prepared essentially by the method of Wagener (1923). A very strong reaction (+++) was obtained in three separate tests on one infected case, whereas 1 ++, 22 +, and 13 negative reactions were obtained on 22 persons with various other infections. With only one known infected case, and not knowing accurately the previous history of the supposed uninfected individuals, the results mean very little, but they suggest that further work along this line might be profitable.

IV. *Trypanosomiasis*

The author has been unable to find any extensive work on the intradermal test in trypanosomiasis. There are, however, a few papers on the intrapalpebral and ophthalmic reactions. The former is essentially an intradermal test of the eyelid, and the latter involves the instillation of material into the conjunctival sac. Admitting the contention of some immunologists that the three clinical types of skin reactions result from different mechanisms, it is impossible to say, from the available evidence, to which type of hypersensitiveness a given ophthalmic reaction belongs.

As early as 1908, Uhlenhuth and Woithe reported negative results with both cutaneous and ophthalmic tests in rabbits infected with *T. equiperdum.* Lanfranchi (1915), using as test material aqueous, alcohol, glycerin, ether, or chloroform extracts of isolated trypanosomes, stated that *T. evansi* infections in dogs could not only be diagnosed with the ophthalmic test but could be differentiated from *T. brucei* infections, and that similar results could be obtained in horses with both the ophthalmic and intrapalpebral reactions. Hornby (1919) failed to repeat these results, but Lanfranchi and Sani (1921), continuing the work, studied horses infected with *T. equiperdum.* Their test material consisted of *T. brucei,* isolated from

blood elements by a hemolytic serum and centrifugation and emulsified in glycerin (1:3) or in glycerin diluted with distilled water or saline; 0.5 c.c. constituted the dose. Infected animals showed almost the same symptoms as those obtained in the mallein reaction in glanders. Swelling of the eyelids appeared by the third or fourth hour, reached its maximum between the twelfth and eighteenth hours, and remained appreciable until the thirty-sixth hour with accompanying symptoms of a slight rise in temperature, photophobia, and lacrymation. Infected animals, during the course of treatment or at its end, showed less pronounced fever and less marked local edema, which reached its maximum around the tenth or eleventh hour and disappeared between the twentieth and twenty-fourth hours. Healthy animals showed local symptoms comparable to those of treated animals, but no general symptoms. The authors suggested that even better results might be obtained with test materials from the homologous *T. equiperdum.*

The intrapalpebral test was again studied by van Saceghem (1922 c) who prepared his test material, which he termed "trypanoleine," by placing 5-10 c.c. of defibrinated blood containing large numbers of trypanosomes on slants of Ponselle medium (2 per cent unwashed agar in tap water), leaving it at room temperature three days during which the blood was laked by the hypotonic medium, collecting the deep red liquid and mixing with a similar amount of a mixture of glycerin and physiological saline (equal parts) and adding a few drops of carbolic acid as a preservative. In performing the test, 0.1 c.c. of this material was injected intrapalpebrally. A positive test consisted in the occurrence in about three hours of lacrymation and an intense painful edema which might persist several hours. He considered the test infallible but gave no account of controls. In trying to repeat his results, van den Branden and van Hoof (1923) concluded differently, because control material consisting of blood of normal guinea-pigs gave equal reactions in four of the six cases of human sleeping sickness tested. Velu, Barotte, Balozet and Bigot (1924) similarly found it of no value in the diagnosis of animal trypanosomiases.

V. *Schistosomiasis*

With the intense interest that has been displayed in the development of serological tests for schistosomiasis, it is strange that so little work has been done on cutaneous tests. As far as the author is aware the only published results are those of N. H. Fairley and Williams

(1927) who obtained, in eight persons infected with *S. hæmatobium,* seven positive reactions of the immediate type, of which three also gave delayed reactions. The authors believed the test valuable to diagnose the presence of infection but not of cure, because two patients regarded as cured by both clinical and serological criteria still reacted. This is of course not unexpected in view of findings with other helminths. Their test material was prepared from powdered infected "livers" of snails (see work of N. H. Fairley in Chapter II). The present author, Hoffman and Cook, in connection with work on precipitins in intestinal schistosomiasis, obtained positive skin reactions of the delayed type in rabbits infected with *S. mansoni,* but this work is in the preliminary stage and has not yet been published.

VI. *Hydatid Disease*

The first intradermal test for this disease was devised by Casoni (1911-1912) who used a carbolized filtered cyst fluid from a sheep (one drop pure phenol to 20 c.c. fluid). In twenty-five tested persons, of whom seven were proved to be infected, the injection of 0.5 c.c. of this fluid was followed by a delayed type of reaction in from three to twelve hours in eight cases. Subsequently, Gasbarrini (1919) reported eleven positives in twelve patients (the exception had a suppurating cyst) and negatives in a number of uninfected persons; and Testi and Zoli (1919) obtained positives in the thirty patients tested. Luridiana (1920 and 1921), using cyst fluid preserved with chloroform, found 81.5 per cent positives in twenty-two patients and negatives in twenty uninfected controls. Moreover, comparative tests on ten of the infected individuals showed seven positive intradermal, eight positive complement fixation and five positive precipitin tests.

K. D. Fairley (1923) used cyst fluid from man or sheep passed through a Berkefeld filter and stored in ampoules on ice without preservative (reactivity retained for several months). He obtained forty positives in seventy-two proved cases and invariable negatives in uninfected cases. Ithurrat and Calcagno (1923) found such cyst fluids of animal origin preferable to those from man. Goudsmit (1924) advised the withdrawal of the fluid sterilely with the addition of 0.5 per cent phenol if it were still sterile after five or six days. In the same year, Mogena (1924) by injecting 0.5 c.c. of bovine or 0.1 c.c. human cyst fluid obtained positives in all of fourteen cases, while Trenti (1924) with 3.0 c.c. of either sheep or human fluid

reported seventeen positives out of twenty-one cases. Dew, Kellaway and Williams (1925) found the test gave ninety-two per cent positives, was absolutely specific, and was not inhibited by suppuration or rupture of cysts. They used 0.2 c.c. of pooled filtered cyst fluid from the liver or lungs of sheep without preservative; the fluid kept its potency for six months. Lemaire and Thiodet (1926) felt that they obtained very satisfactory results with a fluid dialyzed in a sac of goldbeater's skin coated with collodion and suspended in a vessel of distilled water in the ice-box (nine hours was as effective as twenty-four or thirty-six hours) and then filtered through a Chamberland candle. With the same material, Lemaire (1926) obtained twenty-one positives out of twenty-four cases. Deusch (1925, see also 1926) obtained positive results in all of nine hydatid cases and negative results in a series of controls, some infected with tapeworms, *Ascaris* or *Enterobius,* others with syphilis. Both Bisbini (1925) and Sette (1925) used 0.5 c.c. of cyst fluid for the test; the latter reported that the reaction appeared after several hours in 87.5 per cent of the cases tested.

Most recent investigators consider the intradermal test more reliable than either complement fixation or the precipitin reaction. Its superiority is stressed by Pontano (1920), Luridiana (1921), Ithurrat and Calcagno (1922 and 1923), Trenti (1924), Mogena (1924), Goudsmit (1924), Deusch (1925), Dew, Kellaway and Williams (1925), Sette (1925), Hercus (1926), Lemaire and Thiodet (1926 b), and Ichok (1927). On the other hand, del Torto (1922), working with fourteen cases, found complement fixation positive in 86 per cent and the intradermal reaction in only 68 per cent. Furthermore, P. and A. Escudero and Peco (1926) stated that the intradermal test for hydatid loses its value after the first injection, since subsequent tests are positive; and they believed complement fixation to be the only specific test. Other investigators have likewise emphasized the possibility of establishing skin sensitivity by one or more intracutaneous tests. Botteri (1922) temporarily (for a few weeks) sensitized persons by the subcutaneous injection of hydatid fluid. Serigós (1926) stated that the test should be carried out in the skin nearest the suspected site of the cyst.

It becomes of considerable theoretical and even practical importance to classify the type of skin reaction involved in the intradermal tests for hydatid disease. Of the three tests outlined in the introduction there seems little need to consider local anaphylaxis (Arthus phenomenon) since there appears to be no evidence that typical local anaphylaxis, with its possibility of passive transfer, correspondence

with precipitins and general anaphylactic nature, occurs in the skin of man.* The other two types of skin reaction undoubtedly occur in hydatid and have been more or less indiscriminately classified as the Casoni reaction. Indeed, in many cases it is not clear which reaction was being studied. Casoni, however, stated that the reaction became positive in three to twelve hours; he was, therefore, dealing with the delayed reaction. So were most of the earlier workers. Bacigalupo (1918), Gasbarrini (1919), and K. D. Fairley (1923) also used this reaction. On the other hand, Magath (1921), in testing three infected persons and twenty uninfected controls, used the scratch method and obtained in the three infected persons positive reactions in twenty minutes which subsided in about three hours; he was, therefore, dealing with the immediate type of reaction. Ithurrat and Calcagno (1923) also used this type of reaction and termed it the Ymaz-Lorentz reaction. Dew, Kellaway and Williams (1925) sharply delimited two phases of the skin reaction and described the appearance of a wheal in twenty minutes surrounded by a zone of erythema which faded in one hour, and subsequently, a second erythema and deep induration (eight to ten hours) which might last twenty-four to seventy-two hours. (See also Dew and Williams, 1924, and Kellaway, 1925.) They laid particular stress on the diagnostic value of the first phase, that is, the immediate reaction. Goudsmit (1924) agreed with them in this.

At the present time it is impossible to say just what the two types of intradermal tests mean in hydatid. As I have pointed out in the introduction to this chapter, the atopic or immediate reaction in hay-fever and asthma is only elicited in persons with a peculiar inherited complex. Coca has pointed out, and I shall discuss, in relation to similar tests with the intestinal worms, the fact that this type of hypersensitiveness to worms differs from that to pollens, etc., in that all people seem capable of developing it. This is borne out in hydatid by the fact that recent investigators find the test to be of practical importance diagnostically; this could hardly be true in a long series of cases unless most people could develop the hypersensitiveness. One puzzling fact is that, in general, available evidence indicates that once a person has acquired this type of hypersensitiveness it is retained

* At the present time, this classification seems warranted by the lack of evidence of a true Arthus phenomenon in man and the similarity between the tuberculin and the delayed reaction in hydatid. Contrary to this view it must be noted, however, that Botteri (1922), apparently working with the delayed type of reaction, has passively sensitized two persons with the serums from sensitive patients (in large doses of 300 gm. subcutaneously and 135 gm. intravenously).

for a long time, possibly throughout life. If this holds for hydatid, it would invalidate the use of the immediate wheal as a test for recurrent infections. In this connection Kellaway (1925) believed the immediate wheal to be the best criterion for infection, but pointed out that the skin sensitivity lasts for many years and is, therefore, not a test for recurrence. The use of the immediate reaction is further complicated by the fact that persons show a similar type of hypersensitiveness to various animal proteins. To rule out this possibility where cyst fluids of animal origin are used, suitable control tests must be included along with the actual test. (For a discussion of this, see Kellaway, 1925.) Practically all investigators working with this type of hypersensitiveness have found no correlation between precipitins or complement-fixing antibodies and skin sensitivity. (See the work of Coventry and the author on intestinal helminths, page 194.) This lack of correspondence is discussed later.

Compared with the immediate reaction, the delayed reaction would be expected to disappear much more quickly after removal or destruction of the parasite. Very probably, then, it could be used to detect recurrences. Furthermore, when cyst fluids of animal origin are used, it would not be complicated by hypersensitiveness to the host proteins. In many infections a delayed type of reaction is elicited in an individual many days before humoral antibodies can be demonstrated in the blood (see Bachman's work on *Trichinella,* page 187); and even after both are present, there may be many discrepancies in their respective strengths (see Coventry's work on *Ascaris,* page 195).

The fact that there need be no connection between the presence of either the immediate or delayed skin reaction and humoral antibodies raises the question of the correspondence of the skin tests and various serological tests. K. D. Fairley (1923) found that although complement fixation and precipitin tests corresponded, the intradermal reaction (of the delayed type) may not correspond with either serological test, and that the Casoni test may be positive when the others are negative. Similarly, Kellaway (1925) noted cases which were positive with the immediate type of intradermal test, but negative with complement fixation and the precipitin test.

In using either the immediate or delayed type of reaction, a certain number of pseudo-positives occur. These can best be ruled out by injecting, simultaneously with the tests, control solutions, i.e., saline or, where the hydatid fluid is preserved, saline plus the same concentration of preservative. Furthermore, as has been indicated previously, where the immediate type of reaction is used, which involves the possibility of sensitivity to host proteins, it is often

desirable to inject a control solution of proteins from the animal furnishing the hydatid fluid. (As a kind of indicator of sensitivity, some authors use such materials as 1 per cent peptone solution.)

The nature of the exciting agent in the production of positive skin tests in hydatid disease has been the subject of several recent investigations. In comparing different fractions of hydatid fluid Botteri (1923) found that the isolated euglobulin was the most active in eliciting the skin reaction, the pseudoglobulin somewhat weaker and the albumen practically non-reactive. Protein-free lipoids were without activity. Lemaire and Thiodet (1926) found that heating to 55-75° C. for thirty minutes did not destroy the reactivity of the fluid, dialyzed and filtered as described previously. Lemaire, Thiodet and Derrien (1926), after various tests of the same dialysate, came to the conclusion that the active material responsible for the Casoni reaction was not a protein, but a toxic polypeptide, and could be precipitated by alcohol and redissolved in saline. Kellaway, Fairley and Williams (1928), however, questioned the non-protein nature of the reactive material, on the ground both of possible leaks in their filters and of the insufficiency of their chemical tests for the presence of proteins. These authors found that the ultrafiltrates from pyroxylin membranes gave no chemical or anaphylactic tests (Dale and Laidlaw's uterine strip) for protein, but, concomitantly, gave no complement fixation with positive serums and no skin tests (immediate type) in sensitive persons. Ultrafiltrates from other kinds of filters gave positive chemical (Spiegler and hemochromogen tests) and anaphylactic tests for protein and gave feeble complement fixation with positive serums and positive intradermal reactions in sensitive persons. Although the pyroxylin membrane prevented the passage of proteins when sheep serum was filtered alone, traces of sheep serum protein were found in filtrates from hydatid fluid. (Cf. the findings of Ransom, Harrison and Couch, 1924, on *Ascaris,* page 190.)

VII. *Trichiniasis*

Attention has already been called to the desirability of an immunological test for the diagnosis of trichiniasis, and to the further fact that the precipitin test (see especially Bachman, 1928), although quite promising for the diagnosis of established infections, gave no promise for early diagnosis because antibodies were not demonstrable before about the thirtieth day of infection. Bachman (1928 b), however, has demonstrated a clearly defined delayed type of skin reaction in twelve guinea-pigs and thirty-five rabbits after less than a week's

TABLE 25

SKIN REACTIONS AND PRECIPITIN TESTS ON RABBITS INFECTED WITH TRICHINELLA * (FROM BACHMAN, 1928 B)

Rabbit number	*Days after infection*	*Time of reading*				*Precipitin titer* †	*Average numbers of larvæ in the muscle per field at autopsy*
		1 hour	*5 hours*	*12 hours*	*48 hours*		
1	1	0	0	++	0		
	2	0	++	+++	0		
	9	++++	++++	++++	+++		
	19	++++	+++	+++	++	0	
	57	+++	+++	+++	+++	3,000	
	75	+++	+++	+++	+++	3,500	6
2	1	+	+	0	0		
	2	0	++	++	0		
	9	+++	+++	++++	0		
	19	++++	++++	++++	++	0	8
3	1	0	+	++	0		
	5	++++	++++	++++	+++		
	16	+++	++++	++++	++	0	
	70	++++	++++	++++	+++	3,000	
	76	++++	++++	++++	+++	2,500	5
4	1	0	+	++	0		
	2	0	0	0	0		
	5	++++	++++	++++	++++		
	16	++	++	++	++	0	
	76	+++	+++	+++	++++	3,500	5
5	1	0	++	++	0		
	2	0	+	+	0		
	5	0	++	++	0		
	16	++++	++++	++++	++	0	
	59	Dead				2,500	6
6	39	++++	++++	+++	0	2,500	
	45	++++	+++	+++	+	3,500	5
7	4	0	0	0	0		
	18	0	0	0	0		
	25	0	++	++	+++		
	35	+++	+++	+++	+++	100	
	91	+	+++	+++	++	2,000	
	98	+	+	++	++	1,500	
	105	+	0	0	+		
	133	++	+	0	0	3,000	15

* Two skin tests, one using Coca's as the diluent for the *Trichinella* powder and the other using 0.01*N* HCl in 0.85 per cent saline, made on each of these rabbits prior to infection were uniformly negative. Control skin tests using the diluent alone and performed at the same time as the skin tests tabulated above, were negative in all cases after five hours.

† The precipitin titer is expressed in terms of the highest dilution of diluent to dry trichina powder which reacted with undiluted antiserum.

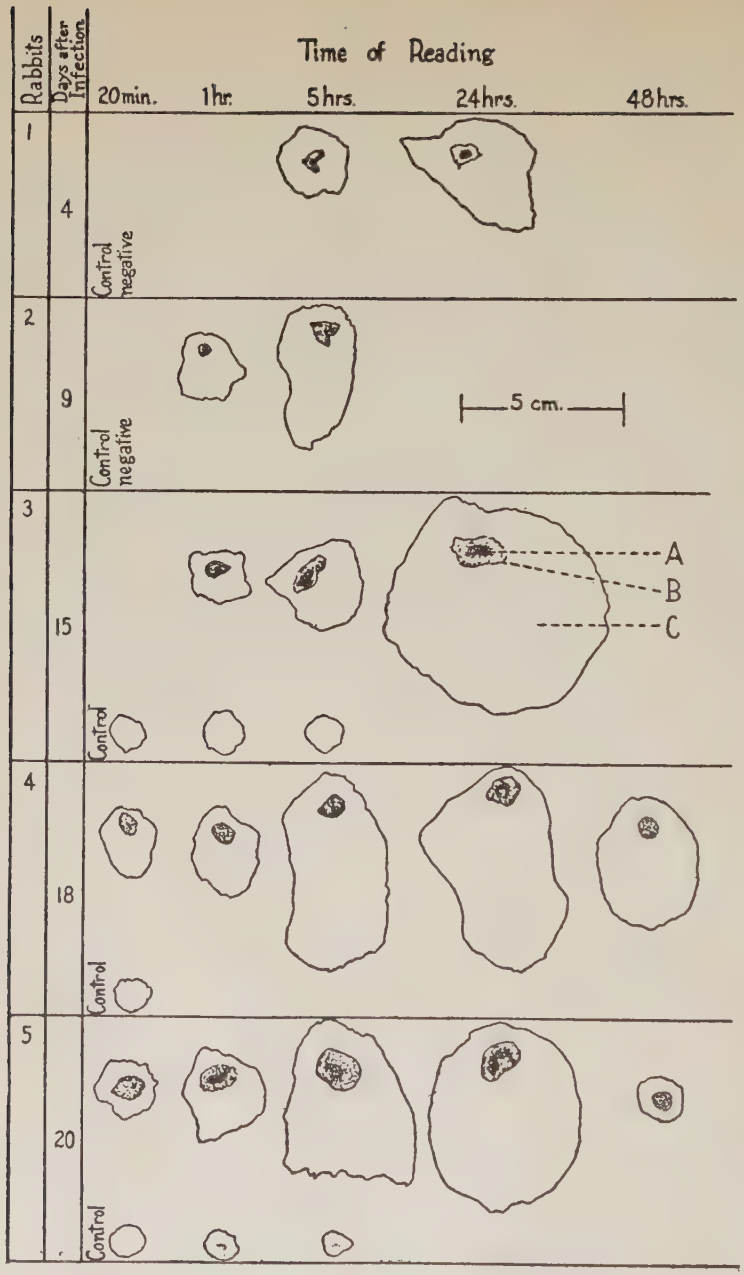


FIG. 21.—Outline drawings of delayed skin reactions, at various intervals after intradermal injections of *Trichinella* protein, in five rabbits injected at four, nine, fifteen, eighteen, and twenty days, respectively, with *Trichinella spiralis*. (After Bachman, 1928 b.) Outlines of controls are given only when they were slightly positive. A, hemorrhagic area; B, hyperemic area; C, edemic area.

infection with *Trichinella,* whereas three normal guinea-pigs and five normal rabbits showed a very weak reaction or none at all. With test materials similar to those used in his precipitin tests, except that the material digested in 0.01*N* HCl did not have to be neutralized before use, he obtained the following reaction: "One hour after the injection, the hemorrhagic area appears bluish red in color; edema is also evident. Five hours after injection, the hemorrhagic area appears darker in color, 0.5 to 1 cm. in diameter, and is surrounded by a hyperemia; well-marked inflammatory edema is also quite evident and necrosis sometimes appears. Twenty-four hours after the injection, the hemorrhagic area appears brownish-red surrounded by a well-marked hyperemia; the inflammatory edemic area may be somewhat enlarged, often reaching a diameter of 3 to 6 cm. and a thickness of 3 to 5 mm. Forty-eight hours after the injection, the hemorrhagic area assumes a scab-like appearance and is often reduced in size and color. The inflammation and edema may have disappeared or become greatly reduced. This is followed by healing and repair."

Figure 21 shows outline drawings of a representative series of delayed skin reactions in five rabbits at varying times after infection, and Table 25 shows the degree of reactivity throughout infections in seven rabbits. It is to be noted that some normal animals after several skin tests gave positive reactions. To show that his serial tests could not be explained on a similar basis, Bachman made only one test on a series of infected animals at varying times and obtained essentially the same results as in his serial tests.

VIII. *Intestinal Helminths*

During the past few years, considerable interest has been focused on the immediate skin reaction among the intestinal helminth infections. Before taking this up it is significant to point out that this reaction represents only one phase of the more general hay-fever-like hypersensitivity which individuals may develop due to contact with worms and which was recognized as early as 1870 by Huber, who reported on the toxic effects of horse and human *Ascaris.* The general symptoms in *Ascaris,* for example, according to Ransom (see Ransom, Harrison and Couch, 1924), who was himself susceptible, are "irritation of the mucous membranes of the eyes, nose, and throat, lacrimation and edema of the eyes, facial edema, sneezing, coughing, swelling of the nasal mucosa, increased nasal and bronchial secretions, painful deglutition, urticaria, asthma, headache, fever, pruritus, tingling and burning sensations, swelling of the fingers, lassitude and

weakness sometimes amounting to prostration." (See also Ransom, 1922.) The skin reaction itself is represented in Figure 22.

Most of the work on skin reactions has been carried out with *Ascaris*. In a very valuable series of investigations on the nature of the exciting principle, Ransom, Harrison and Couch (1924) summarized their results as follows:

"From the results of these experiments, assuming for convenience of discussion that only one substance is involved, it is evident that the substance in aqueous extracts of *Ascaris lumbricoides* that causes the skin reaction in *Ascaris*-sensitive individuals is absent from the globulin fraction, present in the albumen fraction and present in the so-called protein-free filtrate, after the removal of the globulin and albumen fractions by precipitation with ammonium sulphate. It is weakened by oxidation with potassium permanganate so that it no longer produces a skin reaction in all *Ascaris*-sensitive persons. It is not volatile at temperatures between 20° and 100° C. It is thermolabile and is destroyed by exposure to a temperature of about 100° C. for less than an hour but may survive exposure to a temperature as high as 100° C. acting for a period of about fifteen minutes. It is destroyed in the albumen fraction by digestion with pepsin. It is soluble in 50 per cent alcohol. From the acidified protein-free filtrate it is wholly adsorbed by Lloyd's reagent and wholly precipitated by Mayer's solution. It is also wholly adsorbed from the acidified albumen fraction by Lloyd's reagent but has not been recovered from the latter by subsequent treatment with weak alkali, a procedure which releases it from Lloyd's reagent after adsorption from the so-called protein-free filtrate. The question whether it is of protein nature has not yet been answered by our investigations."

Furthermore, in scratch-testing twenty white adults, they obtained four positive reactions, one of which occurred in a person who had been previously unaware of his sensitivity. It is interesting to note, moreover, that although the four positive reactors had been in contact with *Ascaris* during periods of five to twenty-five years with no history of infection, some of the non-reactors had also been in contact with *Ascaris* but had never experienced any disagreeable effects, and several others had a history of infection.

Although not established, the principle involved in the urticarial skin reaction in *Ascaris*-sensitive persons may be the same as that involved in the reaction observed by Weinberg and Julien (1911, 1911 b and 1913), evidenced by edema of the eyelids, congestion of the conjunctiva, lacrymation and other general symptoms following the introduction of body fluid of *Parascaris* into the conjunctival sac. The toxic properties were found to be due to a complex of poisons which were partially volatile, thermostabile (withstanding 120° C. for twenty minutes) and partially soluble in alcohol and in ether. The

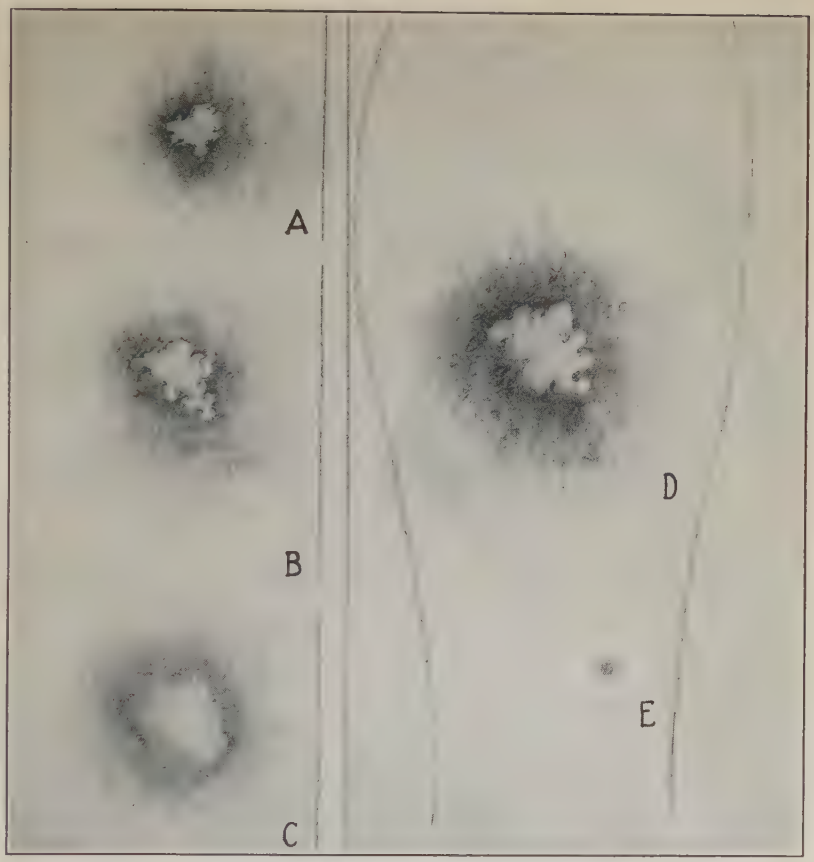


FIG. 22.—D shows an immediate skin reaction resulting from an inoculation (scratch test) of *Ascaris* extract in an individual sensitive to *Ascaris* after a twenty-minute interval; E shows the control following the inoculation of the diluent alone in the same individual after the same interval. A, B and C represent other drawings of the skin reaction at five, ten and sixty minutes, respectively (× about ¼).

discrepancies in the two lists of characteristics may be accounted for, as Ransom, Harrison and Couch pointed out, by the fact that Weinberg and Julien used *Parascaris equorum* and dealt with the body cavity fluid, whereas Ransom, Harrison and Couch used aqueous extracts of the pig ascaris.

Grübel (1924) carried out a total of thirty-five intradermal injections with physiological saline suspensions of ground *Enterobius vermicularis* and *Phthirus pubis,* and found that uninfected persons showed no reaction, while infected persons showed an immediate skin reaction beginning in five minutes after injection and progressing with increased itching and characteristic wheal formation. The parasitic material was reactive after filtration and after heating to the boiling-point.

Fülleborn studied cutaneous reactions in various helminth infections. He (1925, 1926, 1926 b) reported specific immediate reactions in *Strongyloides* patients tested with homologous extracts by the scratch method, and also showed that immediate wheals appeared in hypersensitive persons during the process of the penetration of the larvæ into the skin. Therefore, when he ascertained that such reactions appeared around the anal region of infected persons, he believed it to be due to the penetration of larvæ from feces contaminating the anal region—a process of autoinfection probably responsible for the long duration of such infections. In a later report (1926 c), he concluded that the test is utilizable diagnostically, although its value is somewhat mitigated by the fact that sensitivity may be present for a long time after the cessation of infection. Similarly, he found in *Ascaris* infections that sensitivity lasts at least four years after demonstrable freedom from infection, and concluded that a positive reaction may indicate present or past infection.

As a result of some preliminary skin tests with *Tænia,* Ramsdell (1927) concluded that there is "evidence of the occurrence of a skin sensitization" which persists after the removal of the parasite and which is also manifest in certain other helminthic infections.

Brunner (1928) carried out intradermal tests by the injection method with *Ascaris* and *Diphyllobothrium* on 277 individuals and with *Tænia solium* on twenty. From these he concluded that the reactions were immunologically specific, as indicative of present or past infection, and a valuable aid in the clinical diagnosis of helminthiasis. His data, however, as pointed out by Coventry and the present author (1928), do not seem to warrant these conclusions. Thus, he obtained 19 +++ and 1 + reaction with *Ascaris* extracts in twenty helminth infections (nine hookworm, six *Trichuris,* three *Enterobius,* one

Trichinella and only one *Ascaris*) and 16 +++, 9 ++, 4 + and 30 — among those with negative stools. The positive reactions were accounted for in persons infected with other helminths on the basis of a group reaction and in uninfected persons on the basis of past infection. In trying to elucidate this last point he carried out some tests on children, but here again the tables showed that 4 out of 7 +++ and 86 out of 88 ++ or + reactions were obtained on children with negative stools. The ++ and + reactions he did not consider of diagnostic value, but in view of the individual variation often encountered, this does not seem a legitimate conclusion. Admitting the validity of Brunner's conclusions, it is difficult to see how a reaction which persists after the infection is eliminated and which does not differentiate between various species of nematodes can be of much value in practical diagnosis, especially in the tropics where some past or present nematode infection is well-nigh universal.

The unsuitability of the immediate type of reaction for the practical diagnosis of the intestinal helminths in the tropics is well brought out in a study by Coventry and the author (1928) on natives of Honduras. We used the scratch method with aqueous extracts of the parasites and found that of 130 natives tested with *Ascaris* extracts, 80 per cent gave some grade of positive skin reaction, and of ninety natives, 61 per cent showed anti-*Ascaris* precipitins; that of eighty-four natives tested with hookworm, 80 per cent gave positive skin tests; and that of sixty-four natives tested with *Trichuris,* 25 per cent gave positive skin tests (Table 26). These tests showed no correlation between skin reactions and precipitins nor between *present* infection and either positive skin reactions or precipitins. For example, skin tests with *Ascaris* extracts on sixty-two persons with demonstrable *Ascaris* infection yielded 48 +, 5 ? and 9 — tests, whereas on sixty-eight persons negative for *Ascaris* yielded 57 +, 2 ? and 9 — tests (Table 26). Similar results were obtained with skin tests using hookworm and *Trichuris* extracts. With regard to the precipitin tests, seventy-seven tests (omitting doubtful readings) with serums from persons yielding positive skin tests gave 39 + and 22 — reactions, while sixteen serums from persons yielding negative skin tests gave 10 + and 6 — reactions (Table 27). The lack of correlation between skin test and precipitins has been quite universally recorded.

The lack of correlation between skin reactions and present infections seems best explained by the fact, previously brought out by Fülleborn, that individuals retain their sensitivity after the infection has terminated. In fact, the 80 per cent of positive skin reactions

TABLE 26

RESULTS OF SKIN TESTS WITH ASCARIS EXTRACTS (FROM COVENTRY AND THE AUTHOR, 1928)

Correlation between	*Stool examination* *		*Skin tests* †			*Total*
			+	?	—	
Ascaris skin tests with *Ascaris* and other parasitic infections	A ‡ +	O § +	38	4	4	46
	A +	O —	10	1	5	16
	A —	O +	37	1	8	46
	A —	O —	20	1	1	22
	Total		105	7	18	130
Ascaris skin tests with *Ascaris* and hookworm infections	A +	H ‖ +	26	3	3	32
	A +	H —	22	2	6	30
	A —	H +	22	1	3	26
	A —	H —	35	1	6	42
	Total		105	7	18	130
Ascaris skin tests with *Ascaris* and *Trichuris* infections	A +	T ¶ +	29	3	2	34
	A +	T —	19	2	7	28
	A —	T +	18	0	5	23
	A —	T —	39	2	4	45
	Total		105	7	18	130

* + = Positive stool; — = negative stool.
† + = All grades of positive; ? = slight redness, no wheal; — = negative.
‡ A = Ascaris.
§ O = Other intestinal parasites, viz., *Strongyloides, Enterobius,* tapeworm, *E. histolytica,* hookworm or *Trichuris.*
‖ H = Hookworm.
¶ T = *Trichuris.*

which we obtained in *Ascaris* and hookworm infections by the scratch method probably means 100 per cent reactivity and hence 100 per cent past or present infection, since A. Brown (1922) has found that of seventy-eight persons reacting to proteins by the intradermal-infection method, only 82 per cent were positive by the scratch method. The fact that these data may be interpreted to show 100 per cent infection bears out Coca's contention, referred to in the introduction of this chapter, that the atopic-like condition represented by worm sensitivity is capable of developing in all persons in contradistinction to atopy proper represented by hay-fever which he believes is characterized by an inherited complex.

On the other hand, the two types of reactions appear to be similar as far as local passive transfer is concerned. Prausnitz and Küstner (1921) first illustrated this in the true atopic condition in fish protein sensitivity. Rackemann and Stevens (1927) demonstrated it in *Ascaris*

TABLE 27

CORRELATION OF PRECIPITIN TESTS WITH ASCARIS INFECTIONS AND SKIN TESTS (FROM COVENTRY AND THE AUTHOR, 1928)

Stool examinations *	*Skin tests* †	*Precipitin results* §			*Total*
		+	?	—	
A ‡ +	+	13	3	11	27
	?	3	1	1	5
	—	5	0	3	8
A —	+	26	2	11	39
	?	1	1	0	2
	—	5	1	3	9
Total		53	8	29	90

* + = Positive stool; — = negative stool.
† + = All grades of positive; ? = slight redness, no wheal; — = negative.
‡ A = *Ascaris*.
§ + = All grades of positive; ? = doubtful; — = negative readings.

sensitivity. Thus, two *Ascaris*-sensitive but uninfected individuals who gave typical skin reactions did not show precipitins in their blood but did show "reagins," since normal persons, when first injected intradermally with their serums and twenty-four hours later with *Ascaris* antigen at the same site, showed wheals and urticaria within twenty minutes. Similar results were reported by Brunner (1928) and by Coventry and the author (1928). The results are very striking as the following experiment from the latter paper shows:

Sites on normal person	*Preparatory injections: Serum from sensitive person*	*Test injections*	*Skin test results*		*Test injections*	*Skin test results*	
		24 hrs. later	*5 min.*	*20 min.*	*7 days later*	*5 min.*	*20 min.*
	c.c.	c.c.			c.c.		
1	0.1	0.1 asc ext.	++	++++	0.1 asc ext.	—	—
2 Control.	0.1	0.1 diluent	+?	+	0.1 diluent	—	—
3 Control.	none	0.1 asc ext.	+?	—	0.1 asc ext.	—	—
4 Control.	none	0.1 diluent	+?	+?	0.1 diluent	—	—

The speed of the development of worm hypersensitivity has not been studied to any great extent. It is, therefore, interesting to note its development in one person (cited by Coventry and the author)

who had no history of infection, but began to work with *Ascaris* during March, 1927, and was subsequently tested by the scratch method as follows:

May 4, 1927	+?
May 20, 1927	+?
May 27, 1927	−
July 14, 1927	++
October 21, 1927	++++
January 17, 1928	++++
Repeated tests since	++++

Precipitins to *Ascaris* have never been demonstrated in her blood.

Many of the previous conclusions were verified by Jadassohn (1928) in his very extensive study of the immediate type of reaction to *Ascaris* extracts. Further, he found that in mixtures of serum from sensitive persons and *Ascaris* extracts there is not only a neutralization of the antibody (ability to passively sensitize normal skin), but also of the antigen (ability to excite reaction in sensitive skin). The neutralization of the exciting agent by the antibody is of particular interest because it is contrary to the usual conception of this type of reaction (see page 175).

The foregoing work has dealt exclusively with the immediate type of reaction. The delayed or tuberculin type of reaction will now be considered.

Schröpl (1926), with an extract from *Enterobius* prepared by macerating 0.04 gm. of the fresh worms in 4 c.c. of 96 per cent alcohol, incubating for twenty-four hours, shaking often, filtering and diluting 1 : 10 and 1 : 100 in saline, carried out intradermal tests (controls consisted of the same percentage of alcohol in saline) on thirty-five persons with the following results: fifteen persons giving a history positive for infection or a positive stool examination showed thirteen positive skin tests; seventeen persons with both histories and stools negative, all gave negative tests (three controls were positive). Readings were made on the second day.

Working in this laboratory, Coventry (1929) has made an extensive study of the delayed type of reaction in guinea-pigs and rabbits infected and immunized with extracts of *Ascaris lumbricoides* from the pig. All of her animals had parallel skin tests and precipitin tests performed on them. Her results are too extensive to review in detail, but the following are some of the salient features: Precipitins and positive skin reactions were obtained in both infected and immunized animals. Both the titer of the precipitins and the strength of the skin

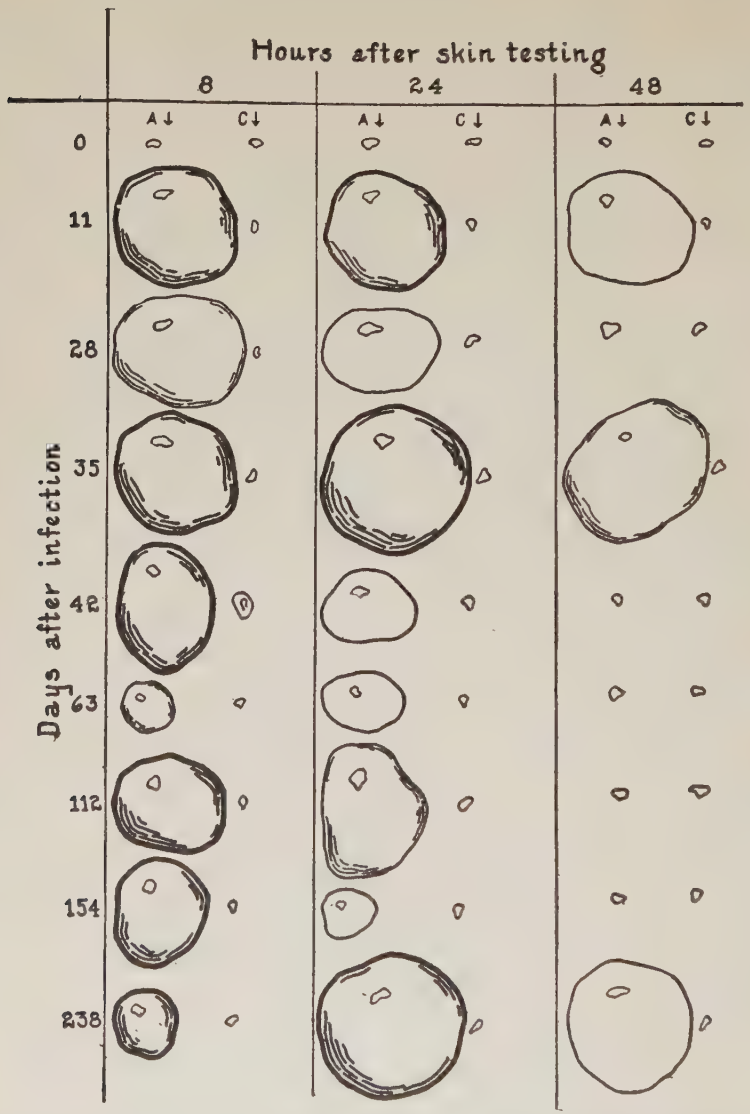


FIG. 23.—Outline drawings of delayed skin reactions at various intervals after intradermal injections of *Ascaris* extract in a rabbit infected with *Ascaris* from the pig. Heavy shaded, light shaded and light unshaded outlines represent marked, moderate and slight edema and induration, respectively (× 2/3). (From Coventry, 1929.)

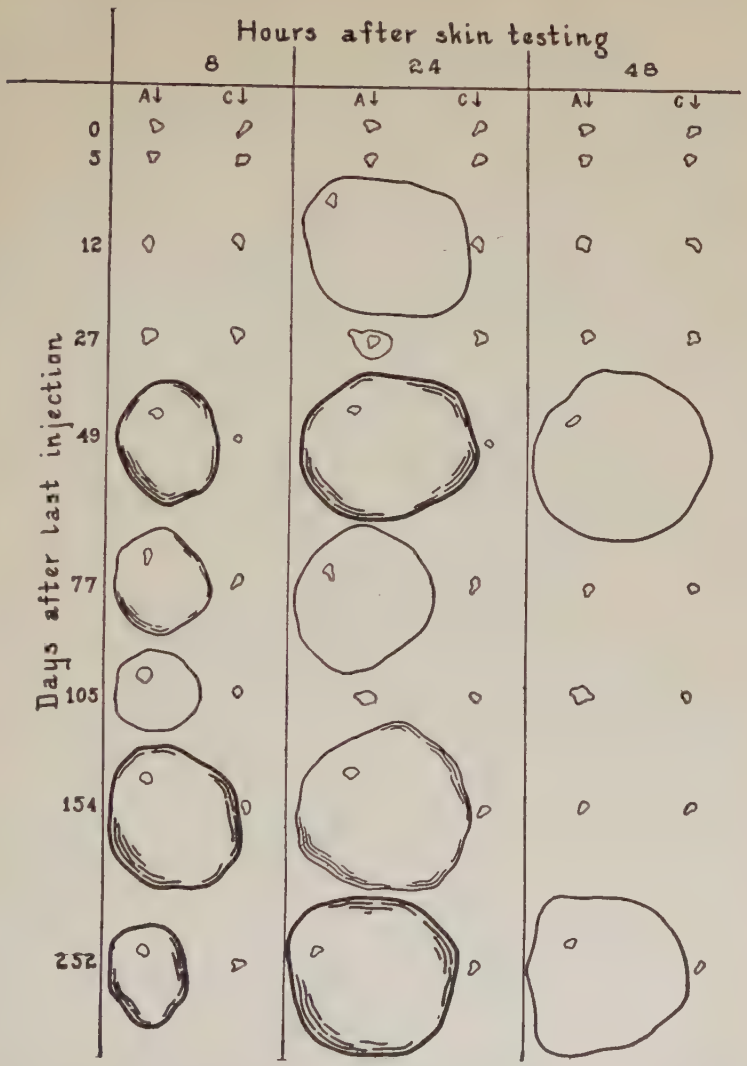


FIG. 24.—Outline drawings of delayed skin reactions at various intervals after intradermal injections of *Ascaris* extract in a rabbit immunized with extracts of *Ascaris* from the pig. Heavy shaded, light shaded and light unshaded outlines indicate marked, moderate and slight edema and induration, respectively (× ⅔). (From Coventry, 1929.)

reaction (particularly the former) showed marked fluctuations throughout the course of the study, but there was no correlation between the fluctuations of the two. In one infected and two immunized guinea-pigs skin reactions occurred, but no precipitins were ever demonstrated. The time of appearance of the two responses varied considerably. In infected rabbits the first precipitins were demonstrable between the sixth and eleventh days, and positive skin tests between the seventh and eleventh days; in infected guinea-pigs the first precipitins were demonstrable between the fourteenth and forty-ninth day (one not at all) and positive skin reactions between the sixth and ninth days; in immunized rabbits, precipitins were found between the fifth and tenth days and positive skin reactions between the seventh and twenty-eighth days after the last immunizing dose; and in immunized guinea-pigs, the precipitins were found between the fifth and ninth days (two not at all) and positive skin tests between the twenty-first and thirty-fifth days. Figure 23 shows a series of outline drawings of skin reactions in a rabbit throughout an infection with *Ascaris,* and Figure 24 a similar series after immunization with *Ascaris* extracts.

IX. *Insects*

A number of investigators have noticed the obvious similarity between the so-called protein wheals (immediate reaction) and the wheals that arise on many persons from the bites of various insects. Mention has already been made of Grübel's (1924) preliminary results on the immediate type of skin reaction with extracts of the crablouse *Phthirus pubis.* Boycott (1926) obtained some interesting results on flea-bites. He ascribed the wheals to anaphylaxis (or, in the terminology of the present book, to hypersensitiveness) which followed a sensitization (previous bites) to the protein in the liquid which the insect injects. He cited the case of an individual sensitive to the bite of *Pulex irritans,* but not to *Xenopsylla cheopis* until after she was bitten five times during six weeks. The same results were later obtained with the rabbit flea, *Spilopsyllus cuniculi.* Peacock (1926) also reported various symptoms of hypersensitiveness in an individual on whom lice had fed and who subsequently was injected with a filtered extract of louse feces.

Blacklock and Gordon (1927) found that after a primary penetration of the skin by one of the myiasis-producing fly larvæ, upon a second or third penetration of the same site, an inflamed area often developed in from one to three minutes, whereas a yellow discolora-

tion developed within twenty-four hours. They did not obtain any indication of an ophthalmic reaction or of anaphylaxis, however.

X. *Summary*

In this chapter three clinical and immunological types of skin reaction have been outlined, all of which depend upon a hypersensitive condition of the host. Of these, there is very little evidence that true local anaphylaxis or the Arthus phenomenon occurs as a result of parasitic infection; but unquestionably the immediate reaction (atopy) occurs in man, and the delayed tuberculin-like reaction (hypersensitiveness of infection) occurs in man and animals. These offer many possibilities as diagnostic tests of infection. As neither reaction is necessarily correlated with humoral antibodies, it seems probable that a combination of a skin test with a serological test would disclose a higher number of infections than either alone.

In man, both the immediate and delayed reactions have reached a high degree of perfection for the diagnosis of hydatid disease, most authors maintaining that such reactions are more reliable than the complement fixation or precipitin test. The fact that, in general, the hypersensitivity which gives rise to the immediate type of reaction persists much longer than that which gives rise to the delayed type suggests the unsuitability of the immediate type as a test of recurrence or cure.

Infections of man with intestinal worms quite generally give rise to positive reactions of the immediate type. Just as in hydatid disease, however, the persistence of the reaction makes its use in diagnosis unpromising because of past infection. The delayed type of reaction, on the other hand, might be suitable for diagnosis; it has been found to occur in man infected with *Enterobius* and in guinea-pigs and rabbits with *Ascaris*.

The delayed type of reaction seems promising for the diagnosis of *Leishmania* and *Trichinella* infections.

The intrapalpebral and ophthalmic tests, although their relationships to the foregoing tests are not clear, have been described in certain trypanosome infections; the results, however, need confirmation. Various ophthalmic tests have also been described in *Ascaris* and other helminthic infections of animals.

CHAPTER VI

THE PRODUCTION OF SYMPTOMS BY PARASITES: TOXINS, OTHER POISONOUS SUBSTANCES (ENDOTOXINS) AND HYPERSENSITIVENESS

I. *Introduction*

There can be little doubt that a large amount of the harm done by some parasites may be classified as mechanical injury to the host. Examples of this are the traumatic effects resulting from the migrations of adult and larval worms and the movement of schistosome eggs through the tissues of the host, the obstruction of the intestine by ascarids and probably the plugging of brain capillaries by subtertian malaria. To this category may also be added the deprivation of the host of essential foodstuffs where valuable foodstuffs are appropriated by the parasites, or as the work of Schern (1925) indicates, where certain hosts affected with trypanosomes die of an acute hypoglycemia due to the use of carbohydrates by the parasites. Such effects as these do not come within the scope of the present work. But when all these—and innumerable other examples could be mentioned—have been accounted for, there seems undoubtedly a residue —vague as to extent—of toxic manifestations produced by the absorption of materials from the parasites, which represents one phase of the immunology of the parasitic infections.

The production of symptoms by the absorption of products from parasites is a subject upon which countless papers have appeared, but about its true nature there is little definite or exact information. Classically, the toxic substances formed by disease-producing organisms have been divided into two large groups: the exotoxins or true toxins, excreted by the invading organism, and the endotoxins which are not excreted, but are retained until liberated through the disintegration of the invader.

The true toxins are all characterized by the outstanding fact that animals can be immunized against them with the attendant development in their serums of demonstrable specific antitoxins. In addition, they are very labile and susceptible to heat (being destroyed at 58-65° C.), light, aging, etc.; they are not immediately poisonous, that

is, an appreciable incubation is necessary; and their toxicity may be destroyed without harming their antigenicity, so that the detoxicated toxins (toxoids) may still engender antitoxin. There is no question of their great importance in the production of disease where they are formed. They have been found in certain bacterial diseases (such as diphtheria, tetanus, botulism, etc.), and are formed by higher plants (phytotoxins, such as ricin, crotin, abrin, etc.) and venomous animals (zoötoxins, such as snake venom, insect toxins, etc.). No true toxins have been demonstrated, however, for the majority of the disease-producing organisms, and more specifically, for none of the parasitic organisms as yet, with the single exception of *Sarcocystis,* the systematic position of which is in considerable doubt. (See Wenyon, 1926.) These organisms, some investigators feel, produce their effects by the liberation of endotoxins, a plausible deduction but one far from verified by experimental data.

The endotoxins have been characterized by the fact that, unlike true toxins, they do not stimulate the production of antitoxins in the animal body, but rather such humoral antibodies as complement-fixing antibodies, precipitins, etc., which are obtained with natural proteins in general. They are commonly more heat resistant than exotoxins and deteriorate less rapidly upon standing. Since they can only be obtained through the disintegration of the invading organisms, they are extremely difficult to demonstrate satisfactorily and many of the substances worked on may be assailed as being protein digestive products (split proteins) which, though admittedly toxic, cannot be strictly called *preformed* specific endotoxins.* In fact, it is very much open to question whether the concept of endotoxins as truly specific poisons is justified, and as a corollary, how extensive is their rôle in the production of symptoms. Many of them may turn out to be similar to tuberculin (see page 216), which is apparently a protein or protein product of the invader, the toxicity of which rests predominantly upon previous sensitization and not upon primary toxicity.

With such an unsettled state of affairs as to the nature of the production of symptoms in those cases where a true toxin cannot be demonstrated, I shall not attempt to review the mass of literature dealing with countless experiments where parasitic derivatives have been found to be toxic to experimental animals, but rather shall discuss illustrative examples and point out some of the reasons for not accepting them unconditionally as the etiological basis for the toxic

* For a discussion of various other toxic substances such as aggressins, virulins, anaphylotoxins, etc., the reader is referred to various standard texts on immunology.

manifestations of the parasites. In addition, I shall review in more detail the work on true toxins and on hypersensitiveness, because, when established, these are of unquestioned importance.

At the outset, it is essential to note that all of the work on antibodies demonstrates beyond peradventure that the body absorbs substances to which it responds specifically. To date, substantiated evidence (since it shows that antibodies are stimulated only by proteins) indicates that these are protein in nature; but many other substances may, of course, be absorbed which we, as yet, have no criteria for ascertaining.

II. *Production of True Toxins by Parasites*

As far as is known at present, true toxins are elaborated only by the Sarcosporidia, which may or may not be true parasites. (See Wenyon, 1926.) In addition, there is some evidence, though based on very incomplete data, that true toxins may be formed by some of the worms.

Upon the injection of aqueous and glycerin extracts of the esophagus of sheep, containing cysts of *Sarcocystis tenella,* into the trachea or muscle of the rabbit, L. Pfeiffer (1891) described various reactions which occurred within twelve to forty-eight hours. Laveran and Mesnil (1899) corroborated and extended Pfeiffer's conclusions, using filtered aqueous and glycerin extracts of both fresh and dried *S. tenella.* The minimal lethal dose for rabbits they found to be 0.001 gm. fresh or 0.0002 gm. dried *Sarcocystis* per kilogram. Large doses of the aqueous extracts in the stomach or intestine produced no symptoms. Although very toxic to rabbits, the material was without effect on guinea-pigs, rats, mice, sheep, dogs, chickens, pigeons, frogs, and turtles. The activity of aqueous extracts was destroyed by heating five minutes at 100° C. or twenty minutes at 85° C. and was markedly diminished by heating two hours at 55-57° C. Glycerin extracts were somewhat more stabile. Using the *Sarcocystis* of the llama as material, Rievel and Behrens (1903) came to the conclusion that the poisonous material was an enzyme and that it exhibited a special affinity for the brain of rabbits. They also immunized rabbits to 10 M.L.D. by a series of six injections of increasing amounts spaced two days apart.

Teichmann (1910) extracted dried powder of *Sarcocystis tenella* with physiological saline. Besides experiments on active immunization, he demonstrated a passive transfer of the immunity by injecting a rabbit with 1 c.c. of immune serum in one ear vein and an ordinarily

lethal dose in the other, whereupon the animal survived. The poison of *Sarcocystis* was demonstrated to be a true exotoxin by the work of Teichmann and Braun (1911 b). Their chief conclusions were that the toxin is thermolabile (destroyed after one hour at 60° C.), soluble in physiological saline, and toxic only for rabbits, though the refractiveness of other species is not due to the possession of a natural antitoxin. They not only immunized rabbits (one rabbit developed an immunity to 100 M.L.D.), but obtained an antitoxic serum which exhibited the following characteristics of a true antitoxin: the antitoxic serum when introduced into another rabbit conferred a passive immunity to the toxin; it neutralized the toxin *in vivo* and *in vitro* so that when it and the toxin were injected simultaneously, or mixed *in vitro* prior to injection, the rabbit survived; and the neutralization of toxin by antitoxin followed the well-known law of definite proportions. These observations were largely corroborated by the subsequent work of M'Gowan (1913), Cominotti (1913), and Sato (1926). Without giving details M'Gowan stated that the serum of infected sheep fixed complement in the presence of extracts of infected muscle, while normal sheep serum did not. Cominotti (1913) found precipitins in the serums of rabbits immunized against the dried cysts of *S. tenella.* Among a series of interesting findings Sato developed an anatoxin by treating the toxin of *S. blanchardi* with formaldehyde, with which a potent antitoxic serum could be developed within a short time.

In the extensive work on worms, the immunization of animals with the toxic materials derived from these parasites has been occasionally reported. Although this may be demonstrated by future work to be due to antitoxin production, it is in no way demonstrated by present data. Shimamura and Fujii (1917) have recovered what they term an "albumose-peptone" from the fractionation of the aqueous extracts of *Ascaris lumbricoides* and *Parascaris equorum,* which gave various symptoms of poisoning when injected into horses, guinea-pigs, dogs and rabbits. Mice and rats were refractory. They have named the toxic principle, without further isolating it, "crude askaron" and found it also in *Dirofilaria immitis, Gastrophilus* larvæ *Strongylus vulgaris, Oxyuris equi* and *Trichuris vulpis.* It lacked hemolytic properties, although these were found in the non-toxic ether and alcohol extracts of powdered ascarids. In susceptible animals, the minimum lethal dose when given intravenously was, per kg. body weight, as follows: horse, 0.004 mg.; guinea-pig, 0.8 mg.; dog, 2.0 mg.; and rabbit, 5.0 mg. Among the symptoms noted were various respiratory and nervous disturbances, dilations of the peripheral blood-

vessels, increase in secretory and excretory functions and decreases in temperature and arterial pressure. Profuse lacrymal secretion occurred in a horse with the instillation of a 1:10,000 solution of "crude askaron." According to the authors, horses could be immunized against the product and by intravenous injections could be made to withstand 400 times the lethal dose. In this connection, it is interesting to note that the immunity thus acquired was believed to be cellular and not humoral and no antitoxin was demonstrated. A further study of the nature of "askaron" was made by Shimamura (1925).

Before leaving the question of the active immunization of animals against toxic materials from parasites, it should be pointed out that this process is not to be confused with the transitory refractiveness exhibited by an animal injected with a sublethal dose of parasitic extract and within a few minutes injected with a second ordinarily lethal dose. Thus, according to Simonin (1920), although 20 c.c. of hydatid fluid kills a rabbit when injected intravenously, a preliminary intravenous dose of 1 to 2 c.c. protects a rabbit against an intravenous dose of 20 to 40 c.c. of the same fluid given ten to twelve minutes thereafter. The protection is transitory and disappears in twenty-four hours; is non-specific, because extracts of one species will protect against another; and cannot be passively transferred to another animal. It is probably identical with the phenomenon of skeptophylaxis and can be produced with proteoses.

Another line of work suggestive of toxin production by parasites is the sporadic reference to a skin reaction, which occurs in normal persons or animals when injected with parasitic extracts, but not in infected persons or animals. This seems to parallel the Schick test for antitoxic immunity to diphtheria where the introduction of a small amount of diphtheria toxin intracutaneously into a non-immune is followed by a decided reaction beginning in about twenty-four hours, reaching its height in three or four days and disappearing after several weeks; the reaction is absent in a previously infected or immune person. Thus, Le Bas (1924 b) with extracts of *Diphyllobothrium* and Ramsdell (1927) with *Tænia* extracts obtained some such reaction, but until these are better understood, there is no proof that the failure of some persons to react is due to an antitoxic property in the serum.

A more thorough, but still inconclusive, study was conducted by Weinberg and Julien (1911, 1911 b, and 1913) and Julien (1926) on the ophthalmic reactions in the horse. They found that the perienteric fluid of *Parascaris equorum* (obtained sterilely) was toxic both to its host, the horse, and to laboratory animals. When instilled

into the eye, a clearly defined reaction developed in about two thirds of 256 horses; this was manifested by a well-developed edema of the lids, beginning in five to ten minutes and rapidly increasing, reaching a maximum in about thirty minutes, when the eye closed with an acute congestion of the conjunctiva and abundant lacrymation, and disappearing, generally, in twelve to twenty-four hours. In severe cases the animal might show many general symptoms. These authors attempted to associate it with the action of a toxin and to demonstrate that infected animals would not show it because they had become immunized little by little against the toxic secretions. The most extensive study of this feature was carried out by Julien (1926). Some of his data have been arranged in Table 28. In regard

TABLE 28

OPHTHALMIC REACTION IN HORSES USING THE PERIENTERIC FLUID OF PARASCARIS EQUORUM (DATA FROM JULIEN, 1926)

Ophthalmic reaction	*Ascarids*		*Infected with intestinal parasites other than ascarids*				*Not infected with any intestinal parasite*
	+	—	*Strongylus*	*Œstrids*	*Oxyurids*	*Tænias*	
+	7	161	147	73	7	4	10
—	48	40	67	33	3	3	7

to the ascarid infections, of fifty-five infected horses, seven were positive and forty-eight negative; and of 201 non-infected, 161 were positive and forty negative. Although not a perfect correspondence, Julien suggested that the seven reactions in infected animals might be due to variability in reactivity or to such a low-grade infection that immunity had not been acquired, and that the forty negative reactions in uninfected animals were due either to a natural immunity to the toxin or to previous infection. He also attempted to demonstrate an *in vitro* neutralization of the toxin, but even in the comparatively low dilutions of 1:200, neutralization was obtained completely in only two horses and partially in a large series (as compared with similar dilutions with normal horse serum and saline). Provided there is an antitoxin, then, it is not of very great potency. In fact, since the reaction looks very similar to the ocular reactions accompanying hypersensitivity, discussed in Chapter V, the data might possibly be explained on that basis, especially when it is recalled, on the one hand, that past infection, which is extremely difficult to rule out except under rigidly controlled conditions, may account for the 161 positive

reactions in so-called non-infected horses, and, on the other hand, that insufficient sensitization due to recent or low-grade infections may account for the forty-eight negative reactions in infected horses. Accordingly, Julien's evidence for a true toxin, although suggestive, cannot be unquestionably accepted until it is definitely shown that the reaction is not a case of sensitization to the ascarid proteins.

III. *Production of Other Poisonous Substances (Endotoxins) by Parasites*

The prodigious amount of work in this field has been largely concentrated on the *in vivo* action of so-called "isolated" endotoxins in an effort to parallel the effects of invading organisms. Actually, in most cases, the procedure has amounted to the injection into laboratory animals (generally intravenously) of secretions, excretions or natural fluids of parasites, or various extracts or suspensions of parasites, with a description of resultant symptoms, as evidence of the etiological rôle of such materials in the infections produced by the corresponding organism. Although the evidence may be pertinent and the conclusions correct, serious criticisms can legitimately be leveled at both. In the first place, such mixtures contain foreign proteins and various disintegration products which may explain their toxicity without the additional assumption of specific poisons such as endotoxins. As brought out by Flury (1912) in his comprehensive pharmacological and toxicological study, these mixtures in the case of *Ascaris* contain a complex of active principles which chemically include aldehydes of fatty acids, free fatty acids, alcohols, esters, and two nitrogenous but non-protein substances. In the second place, admitting that such mixtures are undoubtedly toxic, they may never be liberated in actual infections, or if they should be, may never reach the body through the channel used in the experimental procedure. This is notably true of the intravenous injection method. And in the third place, the disturbances produced have not as yet been satisfactorily correlated either with the symptoms shown by the host or the activities displayed by the invading organism.

In this connection, the blood-inhabiting sporozoa deserve especial attention because the correlation between experimental data and actual conditions in infection seems warranted. The introduction of foreign proteins and disintegration products of proteins intravenously is probably exactly what occurs during infections of the blood-inhabiting sporozoa. In malaria, the best known example, this is further indicated by the striking parallelism between the periodic symptoms

and the periodic development of the parasites. Thus, the young parasites enter * the red cells, grow up more or less synchronously at the expense of the cells, and rupture them more or less periodically to liberate new batches of young merozoites. Although various investigators have postulated toxins or endotoxins † to explain the symptoms, others have—and I believe, more justifiably—tried to explain the symptoms on the basis of the physiological effect of the intravenous introduction of the substances known to be liberated by the rupture of the red cell, such as malarial pigment, remains of the red cells (which have for the most part probably been modified by the parasite) and a certain amount of parasite-proteins. The most obvious parallelism is the similarity between malarial paroxysms and anaphylactoid phenomena.§ The latter are elicited by the intravascular injection of a large variety of agents, such as micro-organisms, tissue extracts, colloids, hypertonic solutions, certain salts, etc. (See Karsner, 1928.) The question has been admirably studied by Abrami and Senevet (1919 and 1919 b). They found that in man the physiological symptoms, which are unnoticed and which they term hemoclasis, such as lowered arterial tension, leucopenia, diminution in red cells, etc., precede, sometimes by several hours, the visible symptoms of the "cold stage," and that the sporulation of the parasites corresponds in time with the physiological symptoms. The "cold stage" they consider as a manifestation of the hemoclastic shock, as "chill" in all degrees is a constant symptom of the reaction. These authors attempted to avert the malarial paroxysm in six cases by the intravenous injection of the patient's own serum eight hours before the expected attack. This serum was obtained the previous evening and the dosage was 20 c.c. In two of the cases the attacks occurred as usual, but were somewhat delayed; in three they were milder; and in one there was no attack. The authors believed that the great difficulty here was in exactly forecasting the commencement of hemoclastic shock and hence the time of making the injections.

* The recent work of Ratcliffe (1927 and 1928) leaves little doubt that at least the avian *Plasmodium cathemerium* and the human *P. vivax* and *P. falciparum* are actually intracellular.

† Schilling (1927) terms these "paroxysmal toxins." Also, Collier (1921) believes that although no endotoxins are liberated, "auto-noxigens" are formed during multiplication of the parasites, which stimulate the body to produce auto-noxins which in turn react in the body to cause fever and paroxysms. "Auto-noxins" are defined as substances which, though originating in the body, act harmfully on it.

§ For the relation between anaphylactoid and anaphylactic phenomena, if any, see Karsner (1928).

The similarity between the symptoms of malaria and those following the intravenous injection of foreign proteins has been stressed by several observers (Barr and Du Bois, 1918; Cowie and Calhoun, 1919; and Barr, Cecil and Du Bois, 1922). The last named observers have adduced experimental evidence indicating that the physiological processes in the two are identical. Thus, in both, during the chill, heat production was greatly increased and heat elimination remained approximately at the basal level; after the chill, heat production dropped sharply and heat elimination remained the same; during the stage of continuous high temperature, heat production fell gradually and heat elimination increased; during the chill the respiratory quotient was high, but after the chill, it fell steadily. Kunde, Hall and Gerty (1927) believed that the favorable results obtained by treating paretics with either malaria or the intravenous injection of foreign proteins arise from the same fundamental mechanism.

W. H. Brown (1912 and 1913) suggested that the pigment, which he (1911) and others considered from their evidence to be hematin or closely related to it, is responsible for at least part of the symptoms, since solutions of hematin caused chills and fever. Butterfield and Benedict (1914), however, were unable to agree with this, and recently Warasi (1927) maintained that the pigment may be more closely related to melanin.

In view of the ambiguous nature of the endotoxins and the lack of definite relationships between the experimental data and the production of symptoms, the work will be rather arbitrarily dealt with.

As illustrative of the historical development, one of the best-known examples of a generally recognized endotoxin, the trypanotoxin of trypanosomes, will be briefly reviewed. Among the first investigators to attempt to demonstrate the production of toxic substances in the trypanosome infections, Kanthack, Durham and Blandford (1898) used fresh filtered serum, blood heated to 50° C., blood and serum kept in sterile containers until the death of the parasites, and extracts of organs of animals infected with *T. brucei;* but with none of them did they obtain ill effects. Similar experiments were reported by Laveran and Mesnil (1902) with *T. brucei;* Musgrave and Clegg (1903) with *T. evansi;* by MacNeal (1904), who used cultures of *T. brucei,* killed by subjection to 34° C. for five days; by Thomas and Breinl (1905) with *T. gambiense* (although the latter once or twice obtained results suggestive of toxin formation); and by Mayer (1905), who used isolated *T. brucei,* killed by keeping or by heating to 58° C. and filtered. MacNeal (1904), however, in attempting to immunize guinea-pigs with attenuated cultures obtained some evi-

dence of a toxin, and he suggested that the solution of the parasites in the latter stages of the infection liberated a toxin which accounted for the symptoms and death. This suggestion received some support from the observation of a number of experimenters that subsequent to the injection of a trypanocide which cleared the blood of parasites there were symptoms of illness and a rise in temperature somewhat commensurate with the number of parasites killed. Thus, Leber (1908) observed a slight attack of interstitial keratitis after injecting the anterior chamber of the eye in rabbits with blood from mice infected with *T. brucei* and treated with arsenophenylglycine. (Control solutions of the drug or trypanosomes were ineffective.)

The first clear-cut evidence that trypanosomes might liberate an endotoxin was furnished by Uhlenhuth, Hübener and Woithe (1908). They showed that blood containing numerous *T. equiperdum,* dried, heated to 37° C. or subjected to low temperatures, proved fatal to rats. Beck (1910) used blood of rats heavily infected with *T. gambiense,* diluted with an equal quantity of 0.85 per cent saline and filtered through a Berkefeld or asbestos filter. A dose of 2 c.c. into normal rats caused somnolescence but recovery within a few days, whereas a dose of 1 c.c. of similarly prepared mouse's blood into mice caused somnolescence and generally death in one or two days. Rabbits behaved similarly, while control animals were unaffected. Laveran and Pettit (1911) were unable to repeat Beck's experiments in four mice, but by using comparatively large quantities of saline extracts of dried trypanosomes (*T. brucei* and *T. evansi*), obtained a depression of temperature, convulsive movements, and profound collapse in mice. They ascribed the previous failures to the use of insufficient trypanosome material. Control injections eliminated the saline, blood-cells, or plasma as significant factors. Laveran (1911 d) obtained essentially similar results with *T. gambiense.*

All of the criticisms previously mentioned apply to this work. At the present time the toxic materials which have been used in the experimental work are, so far as the data go, not proved to be endotoxins. Furthermore, they are not definitely known to be actually formed during infection, and even assuming that they are, it is impossible to define their rôle in the production of symptoms in infected animals. In this connection, the results of Novy, De Kruif and Novy (1917) on "anaphylatoxins" from various trypanosome species are particularly illuminating in explaining the toxicity of materials such as extracts of parasites. Recently Ledentu (1928) carried out a number of experiments and concluded both from these and a critical review of the literature that the existence of trypano-

toxins has not been experimentally demonstrated in spite of the fact that the clinical symptoms of trypanosomiasis point to their formation.

A profusion of work on endotoxin-like products has been carried out on the metazoan parasites, especially the helminths. In an excellent résumé, Simonin (1920) considers the experimental, pathological and clinical evidence that helminths elaborate toxic products which are responsible for symptoms. Such toxic products are often loosely spoken of collectively as "worm toxins" and are individually identified by the parasite from which they arise, as, for example, *Ascaris* toxin; but the term *toxin* here carries with it a connotation of endotoxin rather than toxin, as Simonin explains: "Le terme même de 'toxine vermineuse' est loin d'être l'expression d'une notion concrète et, aujourd'hui encore, lorsqu'on parle de *toxin hydatique,* de *toxine ascaridienne* ou de *toxine ankylostomienne,* on envisage bien moins une substance particulière, determinée par des caractères physiques propres ou des modes de réaction chimique permettant de la spécifier, qu'un agent parasitaire auquel on impute les désordres constatés dans la santé de l'organisme envahi."

There have been two main trends in the work: a study of the lethality and general symptoms following injection of worm materials, and a more exact physiological analysis of the effects on particular systems, such as respiration, blood-pressure and the like. As much of it is extremely conflicting, due probably to diverse technical methods, the use of different species, etc., those interested are referred to the original papers and to the admirable and extensive reviews of Simonin (1920) for the worms, and of Phisalix (1920) and Pawlowsky (1927) for both protozoan and metazoan parasites.

The so-called hemotoxins, i.e., hemolysins, anticoagulins and agglutinins, described in extracts of various parasites, merit particular attention. Of these, the hemolysins are the most important because of their practical significance in complement fixation tests where test antigens may be quite hemolytic, and because, although not proven, they may be instrumental in the production of the severe anemias often associated with the helminths. As their etiological significance in the production of symptoms is still so much in question, I have not attempted to give a review of the entire subject but have simply selected some examples from the very thorough review of the literature on helminth hemotoxins by Schwartz (1921). Although there has been a number of papers published on the subject subsequently, his general conclusions as to the nature and action of the substances have remained unaltered.

The first experimental work among the cestodes to indicate that the anemia in *Diphyllobothrium* infections may be due to a hemolytic property of the worms was that of Schaumann and Tallquist (1898), who obtained hemolysis both *in vivo* and *in vitro* with macerated worms from dogs. Supplementary data were added by Tallquist (1907), who found that the hemolysin was closely bound to the cells of the parasite, was only slightly soluble in water and saline, but by means of peptic digestion and alcohol or ether extraction was dissociated from the tissues and went into solution, was thermostabile, non-specific, and did not stimulate the production of antibodies in infected animals.

Dascotte (cited by Weinberg, 1912 b) found that extracts of *Tænia solium* and *T. saginata* dissolved red blood-cells of their host, man, and that the hemolysin was soluble in alcohol and thermostabile (resistant to 100-120° C.). Calamida (1901) obtained a hemolysin from Berkefeld-filtered extracts of *Dipylidium caninum* and *Multiceps multiceps* (from carnivores) against the cells of rabbits and guinea-pigs. Quite the contrary was found by Weinberg (1907) with saline extracts of the horse tapeworms, *Anoplocephala plicata* and *A. perfoliata;* and Tallquist (1907), after working on a number of species, denied the existence of hemolysins in any cestode except *Diphyllobothrium latum.* Schwartz (1921) found a hemolysin in *Thysanosoma actinioides* (from ruminants) which was moderately soluble in saline, completely soluble in alcohol, active at low temperatures, and resistant to boiling. Similar extracts of *Moniezia* were non-hemolytic.

More positive results were reported among the nematodes. As early as 1890 Lussana maintained that he could produce anemia by injecting rabbits with urine from patients suffering with hookworm disease. In 1904 Alessandrini observed that red blood-cells were destroyed if placed in contact with the cervical glands of hookworms. Calmette and Breton (1905) reported that saline extracts of *A. duodenale* were hemolytic to red cells of man, while Liefmann (1905) noted a weak hemolysin in *A. caninum.* Ample confirmation was accorded, thereafter, by Preti (1908) with *A. duodenale;* by Noc (1908) with saline extracts of *Necator americanus* tested against human cells; by Whipple (1909) with concentrated saline extracts of dog and human hookworms tested against human, dog and rat blood (he found, unlike Alessandrini, that the hemolysin was distributed in all parts of the body); by Usami and Mano (1919) and by Schwartz (1921) with *A. caninum* and *Bunostomum phlebotomum* of cattle. The only negative results were observed by Loeb and

Smith (1904) and Loeb and Fleisher (1910) with saline extracts of *A. caninum.*

These hemolysins were found (see foregoing references) by Preti (*A. duodenale*) to be insoluble in saline, soluble in alcohol and ether, liberated by tryptic digestion in water, resistant to boiling for three hours and non-specific; by Whipple (*A. duodenale, A. caninum, N. americanus*) to be susceptible to boiling and non-specific; by Usami and Mano, insoluble in water, soluble in alcohol, ether, and acetone, and thermostabile; and by Schwartz (*A. caninum*) to be soluble in saline, relatively thermostabile (susceptible to boiling), non-specific, inactive at low temperatures and firmly bound to the tissues of the parasite, especially when the latter are dried, which may account for the earlier reports of insolubility in water and saline. Furthermore, Noc found that their activity appeared to be inhibited by serum from normal persons and those recovered from hookworm, but not by those suffering with the disease, while de Blasi (1908) found that the serum from hookworm cases when heated to 56-62° C. was itself hemolytic, but that normal serum, similarly heated, was not.

Among the other nematodes Schimmelpfennig (1902) found the cœlomic fluid of *Parascaris equorum* hemolytic to horse cells. Similar results were reported by Flury (1912), Shimamura and Fujii (1917) and Schwartz (1919 and 1921). Brinda (1914) reported a diminution of hemoglobin and red blood cells in guinea-pigs as the result of injections of saline extracts of ascarids. Negative results were reported by Weinberg (1907), Whipple (1909) and Alessandrini (1913). In *Strongylus,* non-specific hemolysins were reported by Weinberg (1907), Bondouy (1908 and 1910) and Schwartz (1919). Negative results were obtained by Cuillé, Marotel and Panisset (1911) with extracts of an unidentified sheep *Strongylus.* In another strongyle *Hæmonchus contortus,* a weak hemolysin was found by Brumpt and Joyeux. (See Brumpt, 1910.) Garin (1913) found a hemolysin, secreted by the living worms *in vitro* in *Graphidium strigosum* and *Trichostrongylus retortæformis,* which was relatively specific. Whipple (1909) ascertained that hemolysins destructive to the cells of dog and man were present in extracts of *Trichuris;* Garin (1913) confirmed this, but found the cells of rabbits and guinea-pigs unaffected.

Among the trematodes Yagi (1910) found a hemolytic principle in saline extracts of the human blood fluke, *Schistosoma japonicum,* when tested against cells of cattle, sheep, and rabbits. This was corroborated by Yoshimura (1913) in the case of rabbit cells, but human cells appeared unaffected. Guerrini (1908) believed that

Fasciola hepatica secreted a hemolytic principle which could be demonstrated in the blood serum of the host.

In the case of the thorn-headed worm, *Macracanthorhynchus hirudinaceus,* of the hog, Alessandrini (1913) found a hemolysin, in both extracts and cœlomic fluid, which was soluble in water, insoluble in alcohol and thermolabile (destroyed at 55° C.).

This cursory review of the literature indicates rather contradictory results, but some of the negative results may be accounted for in several ways. As pointed out by Schwartz (1921), some are probably due to faulty extraction and some to the inhibitory action of normal serum. In any case, the valuable series of publications by Schwartz (1919, 1920 b, culminating in his paper of 1921), leave little doubt that there are: (1) hemolysins in extracts of *Ascaris lumbricoides, Ancylostoma caninum, Bunostomum phlebotomum* (cattle hookworm) and the cestode *Thysanosoma actinioides;* (2) weak hemolysins in saline extracts of *Hæmonchus contortus* and *Trichuris vulpis;* and (3) no hemolysins in a species of *Moniezia.* His work was carried out under carefully controlled conditions.

The data on the chemical nature of these hemolysins are conflicting. Although some investigators (E. S. Faust and Tallquist, 1907; E. S. Faust, 1908; Schmincke and Flury, 1911; and Flury, 1912) believed the active principle to be oleic acid, or at least associated with unsaturated fatty acids (Flury, 1912), and brought forward supporting evidence, others (Bondouy, 1910; Csonka, 1918; Beumer, 1919; and Schwartz, 1921) opposed the view by showing that the hemolytic principle was insoluble in ether and soluble in alcohol.

Because of his own and previous work, Schwartz (1921) feels that the worm hemolysins have many characteristics in common with the hemolysins from both bacteria and normal tissue, but, in general, are unlike the immune hemolysins. He gives the following characteristics: (1) They are usually non-specific, although the cells of different species often exhibit different degrees of resistance to their action. (2) They exhibit wide divergencies in regard to their thermolability, some being thermostabile (resembling tissue extracts), others being thermolabile (resembling bacterial hemolysins). (3) If a small series of experiments by Garin (1913) on the nematode *Graphidium strigosum* be excluded, when their activity is destroyed by heat, it cannot be reactivated by normal serum (complement). (4) Their activity is inhibited by normal serum. (5) They are generally soluble in alcohol and ether. Among the nematodes and in *D. latum,* they are closely bound to the tissues of the worm, and are therefore

but slightly soluble in water unless digested by means of pepsin, alcohol, or ether. And (6), the nematode hemolysins resemble bacterial hemolysins in being inhibited at 8° C., while the cestode hemolysins resemble tissue lysins in not being inhibited at this temperature. Wells (1925) has pointed out that the bacterial hemolysins may simply be toxins with a particular affinity for red cells since antitoxic serums have been obtained for some, but that it is uncertain how much of the effect of the antitoxic serum may be due to antitoxin and how much to cholesterol in the serum. He also noted that bacteria produce various non-antigenic hemolytic substances such as bases and acids. To this group the tissue hemolytic substances belong and probably represent decomposition products (e.g., fatty acids).

Such forms as hookworm, which feed on blood, might more or less be expected to secrete an anticoagulin. Loeb and Smith (1904 and 1904 b), Loeb (1906) and Loeb and Fleisher (1910), found that inhibitory effects on the coagulation of dog's blood could be produced *in vitro* by saline extracts of *Ancylostoma,* but that they varied with the samples of blood—in one case lasting for twenty-four hours. Furthermore, these effects were exhibited by extracts of the anterior but not the posterior half of the worms, and although diminished, were not destroyed entirely by boiling fifteen minutes. Although Liefmann (1905) questioned this work, the data by Loeb and his collaborators seem convincing. Similar results were obtained with extracts of *Strongylus* from the horse by Weinberg (1907, 1912 c) on horse blood; and with body fluids of ascarids by Leroy (1910) on dog blood, and by Flury (1912) on dog and human blood. Negative results in dog ascarids were reported by Loeb and Smith (1904), and by Weil and Boyé (1910) in *in vivo* experiments in rabbits.

Schwartz (1921 and 1921 b) made a detailed study of the action of physiological saline extracts of the following nematodes: *Strongylus vulgaris, S. edentatus, A. caninum, Bunostomum phlebotomum, B. trigonocephalum, Stephanurus dentatus, Œsophagostomum columbianum, Dictyocaulus filaria, Hæmonchus contortus, Ascaris lumbricoides, Parascaris equorum* and *Toxocara sp.* Specimens were washed, dried and pulverized. In most cases 0.1 gm. of powder was added to 1 c.c. of physiological saline, allowed to stand at about 10° C. for twenty-four hours and then filtered. To test the anticoagulative property of the resulting extract, it was mixed with equal quantities of freshly drawn blood. As a control, salt solution was added to blood in the same proportions. Schwartz by this work confirmed the results of Weinberg with *Strongylus* and of Flury with

Ascaris, but obtained only weak anticoagulins for *A. caninum,* which he stated may have been due to his testing rabbit instead of dog blood. Some species gave negative results. In general he concluded that, "so far as present knowledge goes, nematodes which contain substances that inhibit coagulation of the blood to a marked degree are zoölogically related, belonging to the family Strongylidae, the members of which have a buccal capsule adapted to lacerating the intestinal mucosa."

Little is known of the nature of these anticoagulins. According to Loeb and Smith (1904), they resist boiling. Schwartz (1921 b) feels that they are not merely mixtures of proteins, but specific anticoagulins probably related to hirudin and certain snake venoms, and that their effects cannot be ascribed to such things as solutions of proteoses, trypsin, pepsin, or extracts of tissues, which retard the coagulation of blood when injected into animals, but have been shown not to retard coagulation *in vitro.*

Certain agglutinating properties of worm extracts, according to Schwartz (1921), are also included under the term hemotoxins. For example, physiological saline extracts of *Ascaris lumbricoides* agglutinated rabbit red cells, occasionally sheep cells, but never hog cells. Unlike the lipoidal hemolysins, he found that this principle was only slightly soluble in ether and alcohol, had a relative specificity, was filterable, was not injured by heating at 56-60° C. for thirty minutes, and was active at low temperatures. Tallquist (1907) also found a hemagglutinin in *Diphyllobothrium latum,* but his principle was soluble in water, was non-specific, was thermolabile and could not withstand a temperature of 55° C. for thirty minutes.

This work, when reviewed critically, does not clarify many of the difficulties. It is, of course, by no means improbable that some of the toxic elements from time to time may be liberated by the disintegration of worms, particularly of the tapeworm, *Diphyllobothrium latum.* As to whether they circulate in the host, Schwartz, who favors the view, cites the following evidence: (1) Worm infestations lead to the formation of definite antibodies demonstrable in the serum of the host. (2) The work of Guerrini (1908) indicated the presence of hemolysins in the serum of hosts infested with *Fasciola hepatica* and was confirmed by the results of de Blasi (1908) with *Ancylostoma duodenale.* (3) Weinberg (1908) found iron granules in the mononuclear leucocytes and the organs, especially the spleen and liver, of thirty-two horses infested with *Strongylus* and thirty monkeys infested with *Œsophagostomum.* Later, the same author (according to Schwartz) obtained pigmentation of the spleen in guinea-pigs

which were injected with extracts of *Strongylus*. Such granules were interpreted as disintegration products of red cells, ingested by the leucocytes and arrested in certain organs—a process which occurs in animals harboring hemotoxin-producing parasitic worms.

IV. *The Rôle of Hypersensitiveness in the Production of Symptoms*

Without definitely affirming or denying the rôle of the various endotoxin-like substances obtained from parasites in the production of symptoms, the present author feels that a much more promising field in this connection may be the study of various hypersensitive states produced in the host by the parasite, with the idea of correlating them with toxicity. Thus, the proteins of the parasite may be relatively harmless to the normal host, but may become highly toxic owing to the sensitization of the host by infection.

Such a condition is best exemplified by tuberculin, the material obtained from tubercle bacilli or from cultures in which they have grown and which a number of authors have considered either an endotoxin or an endotoxin-like substance. Since its discovery in 1891 by Koch, it has been known to be comparatively innocuous to normal animals, but highly toxic to tuberculous ones. Recently, Seibert (1928) has isolated a crystalline protein from tuberculin which after purification by fourteen successive recrystallizations gave all the biological reactions of tuberculin and showed the usual antigenic characteristics of a native protein. The poisonous principle, tuberculin, then, seems to be a protein or protein product of the bacteria, which, although primarily toxic to some extent, is far more potent after infection has sensitized the body to it. * The phenomenon of hypersensitivity in tuberculous infections is further brought out by the reaction of normal and infected animals to tubercle bacilli. If living tubercle bacilli are introduced into an uninfected host (whether man or experimental animal), there is a comparatively slow tissue response (nodular tubercle) which is sharply delimited from the surrounding tissue and which shows no signs of inflammation. If introduced into a previously infected host (after a sufficient lapse of time for the body to become hypersensitive), the reaction is extremely rapid, diffuse, inflammatory, and exudative in character, and old foci from previous infections may "flare up" with an acute inflammatory reac-

* Seibert (1928) has found that the M.L.D. of the protein, purified by the ammonium sulphate precipitation method, when injected intraperitoneally is 0.1-0.15 gm. for a normal guinea-pig, whereas it is only 0.002-0.005 gm. for a tuberculous (sensitized) guinea-pig.

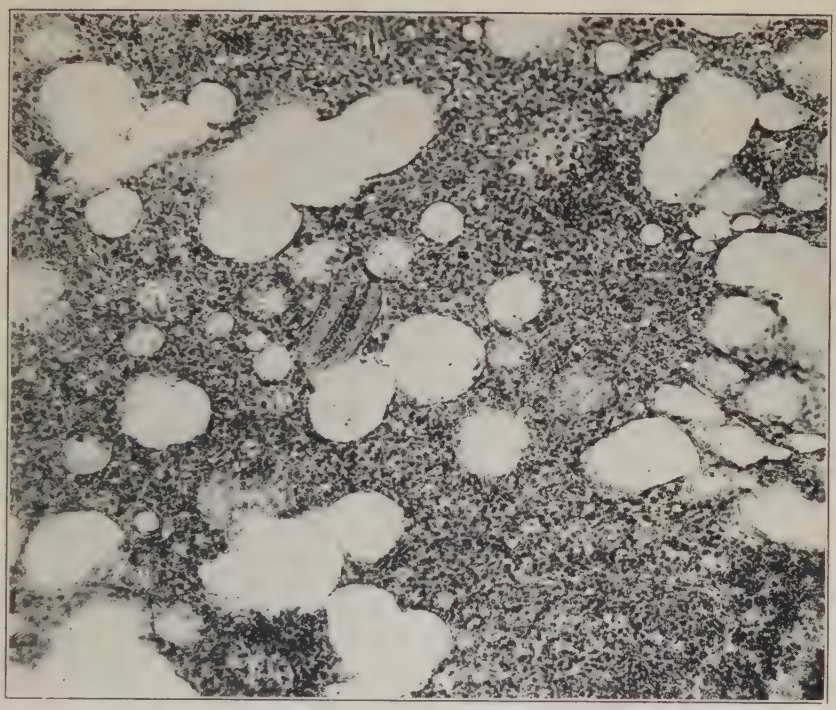


FIG. 25.—Low-power field of a section through guinea-pig lung in the region of an *Ascaris* larva. Guinea-pig previously sensitized with *Ascaris* powder. Proliferation and infiltration of inflammatory cells consisting predominantly of histiocytes and eosinophiles; also edema and hemorrhage. The inflammatory reaction is localized in general around the larva (× 130). (From manuscript by Cannon and the author.)

tion. (See Krause, 1927.) The very intensity of the defense reaction in the infected, sensitized animal may kill it. There can be no doubt that an infected animal responds defensively to the tubercle bacilli more quickly and effectively than does the uninfected, but if the organisms are placed in sites where they are distributed over the body or if the dosage is great enough, the very violence of the defense reaction kills the host.

Some interesting work in this field has already been done on the parasites. Most workers have included all of the hypersensitive states to parasitic proteins under the term anaphylaxis. In this discussion, however, anaphylaxis will be limited to the restricted sense of the term (see page 11) and the relation of it to the other clinical forms of hypersensitiveness will be left *sub judice*. The literature may conveniently be examined with respect to certain definite questions; (1) Are parasitic proteins efficient anaphylactic antigens? (2) Do parasitic infections give rise to true anaphylaxis? (3) What is the rôle of hypersensitiveness in general?

It seems hardly necessary to emphasize that various extracts of parasites and their fluids are efficient anaphylactic and sensitizing antigens. This is to be expected because they contain proteins foreign to the host. A large amount of work on active anaphylaxis has been carried out with hydatid fluid in rabbits and guinea-pigs by Boidin and Laroche (1910), Ghedini and Zamorani (1910), Weinberg and Ciuca (1913, 1913 b and 1914), Simonin (1920), Parisot and Simonin (1920 b), Deluca (1923) and Kellaway (1928). Similar studies with the cyst fluid of other larval cestodes have been made by Mello (1911), Henry and Ciuca (1912 and 1912 b) and Simonin (1920), with extract of *Tænia* by Simonin (1920), and with perienteric fluid of *Ascaris* by Mello (1911) and Simonin (1920). Passive transfer has also been obtained with the serum of rabbits sensitized with hydatid fluid by Parisot and Simonin (1920 c). The uterine strip method has been used by Kellaway (1928 and 1928 b) with hydatid fluid and *Fasciola*.

The question as to whether parasitic infection gives rise to the anaphylactic mechanism can be most easily attacked experimentally by ascertaining whether the serum from an infected host will passively confer on guinea-pigs an anaphylactic hypersensitivity to extracts of the parasite. Guinea-pigs are selected for this work because they are *par excellence* the best animals in which to observe the classical symptoms of anaphylactic intoxication. The fact that, in general, a precipitating serum will passively sensitize a guinea-pig to the specific antigen would presuppose that such experiments

are possible because precipitins are quite commonly demonstrated in parasitic infections.*

In human hydatid Weinberg and Ciuca (1913 b and 1914) found that serum from a patient infected with hydatid would passively sensitize guinea-pigs to the cyst fluid; they suggested this as a mode of diagnosis. For example, a guinea-pig is given subcutaneously 3 to 5 c.c. of the serum from a patient suspected of having hydatid, and forty-eight to sixty hours later † is given an intravenous injection of hydatid fluid; whereupon, if shock occurs, it has been passively sensitized. It is to be noted that this experimental technique satisfies the general criteria for passive transfer. Briefly, passive transfer, in general, can be demonstrated approximately four hours after intravenous injection, twenty-four hours after intraperitoneal injection and forty-eight hours after subcutaneous injection of suitable serum, persists for three or four days, and gradually disappears within a few days (Karsner, 1928); whereas active anaphylaxis is not manifest until eight to twelve days after injection and reaches its height in about twenty-one days. Weinberg and Ciuca (1914) obtained the following results with serums from persons suspected of having hydatid: (1) of twenty serums giving positive complement fixation, eighteen gave passive transfer; (2) of three serums giving negative complement fixation, two gave passive transfer; and (3) of thirty-six serums from persons erroneously diagnosed, none gave passive transfer. Similar results were described by Henry and Ciuca (1914) for the serums of rabbits infected with the larval form of *Multiceps serialis*.

The fact that parasitic products are efficient anaphylactic antigens and the further fact that serums from infected animals may passively sensitize (i.e., contain the anaphylactic mechanism) guinea-pigs does not, as some authors seem to believe, demonstrate that anaphylaxis actually occurs in infected hosts. It may, paradoxically, demonstrate immunity. Thus, one animal after a single dose of foreign protein may be anaphylactically sensitive and its serum in

* See Karsner (1928) for a discussion of the relation between precipitins and anaphylaxis.

† Some authors maintain that the injection of antiserum and antigen simultaneously or in mixture will produce anaphylaxis, but most writers would classify such reactions as anaphylactoid and feel that for true passive sensitization some time must elapse between the introduction of the antiserum and the antigen. Caronia (1913 b) has devised what he terms an anaphylactic test for kala-azar, in which a mixture of infected patient's serum and cultural flagellates is supposed to produce anaphylaxis upon intravenous injection into guinea-pigs.

large doses will confer passive sensitivity; but another animal after repeated doses of the same protein will be immunized, rather than anaphylactically sensitized, and yet its serum in *small doses* will also confer passive sensitivity. This strikingly emphasizes the point that acquired hypersensitiveness and acquired immunity are qualitatively similar, but quantitatively different. In other words, whether or not an animal is immune or sensitive may be a question of the relative amounts of antibodies in the serum. Some authors have even gone so far as to maintain that in man the anaphylactic mechanism may occur, but that the true anaphylactic shock cannot be somehow initiated. (See Coca, 1928).

The next question to be considered is, Does true anaphylaxis occur as a result of a sensitization by past or present parasitic infection? As just pointed out, some immunologists doubt that true anaphylactic shock takes place in man. Others feel that to be the only way of explaining the sudden deaths resulting from the spilling of hydatid fluid during rupture or surgical puncture of the cysts (Humphrey, 1887; Achard, 1888; and Dévé, 1910). Botteri (1922) also believes that anaphylactic symptoms follow the intravenous injection of hydatid fluid into persons sensitized either artificially or by injection, and that such persons can be desensitized. Weinberg and Julien (1911) and Julien (1926) believe anaphylaxis to be responsible for the occasional sudden death of horses upon the instillation into the eye of a small quantity of perienteric fluid of *Parascaris*. Graetz (1912), on the other hand, believes that anaphylaxis does not occur in hosts infected with *Echinococcus* because the protein of the cyst fluid had been derived from the host, but that the shock is due to primary toxicity of the proteins which were probably modified and would, therefore, resemble anaphylotoxins. Undoubtedly, however, there must be proteins in hydatid fluid which are peculiar to the parasite, because Weinberg and Ciuca showed that cyst fluids of various origins contained a common protein. They found that guinea-pigs sensitized with sheep cyst fluid could be later shocked not only with homologous fluids, but also with cyst fluids from man, hog and beef.

As to whether parasitic infection actually sensitizes an animal so that a later injection of parasitic material will produce anaphylaxis while the host is still infected, the experiments of Van Es and Schalk (1917) are apropos. Previous to their work, K. R. and R. Seyderhelm (1914, 1914 b) had obtained a toxic material which they termed *"œstrin"* by triturating *Gastrophilus* larvæ in saline. Since it produced marked symptoms upon intravenous injection into horses,

they believed it to be the etiological agent of pernicious anemia of horses. Van Es and Schalk were able to duplicate many of the immediate symptoms described by the Seyderhelms as resulting from the intravenous injection of saline extracts in suspensions of *Gastrophilus*. They soon began to suspect, however, that the symptoms had nothing to do with pernicious anemia, but were anaphylactic and depended upon a previous sensitization of their horses by infection. To settle this question, they injected six colts intravenously with *Gastrophilus* extracts. Of these, four reacted, two did not. In line with their contention, two of the reactors were infected and the two non-reactors were uninfected, while the two uninfected reactors they plausibly explained on the ground that they were very young and may have been passively sensitized from their mother's milk. Similar experiments with several ascarids and tapeworms in dogs, while not conclusive, strongly supported the idea that animals may be anaphylactically sensitized by infection so that a subsequent injection of the parasitic protein produces anaphylactic shock. All of this work was carried out under conditions where it was extremely difficult to rule out past infection, so that it should be repeated under carefully controlled conditions with parasite-free hosts.

The results of Hadwen and Bruce (1917) indicated strongly that cattle and sheep infected with *Hypoderma bovis, H. lineatum* and *Œstrus ovis* are so sensitized that various grades of anaphylaxis follow the intravenous injection of extracts of the host's own parasites. When these are not fatal, a period of refractiveness ensues. Hypersensitive animals also show ophthalmic reactions.

In this same category of anaphylactic manifestations, many authors include such things as the semiacute syndrome, termed "Rosenfeber" by Brodersen (1919), which occurs in cattle as the result of the improper removal of grubs from the skin. (See also Jensen, 1919, and Hadwen, 1919.)

So far, the question of anaphylaxis, *sensu strictu*, has been considered. Although in the present state of our knowledge, it is impossible to homologize or interrelate anaphylaxis with the other forms of protein hypersensitivity, there can be no doubt that parasitic infection does often result in the host's becoming hypersensitive to the parasitic proteins. Adequate proof is afforded by the entire chapter (Chapter V) on cutaneous tests and is suggested by the work on eosinophilia in parasitic infections (page 227). Not only is the hypersensitivity evident throughout the course of many infections, but it often persists a long time after recovery from infection.

Investigators have repeatedly suggested that such hypersensitivity

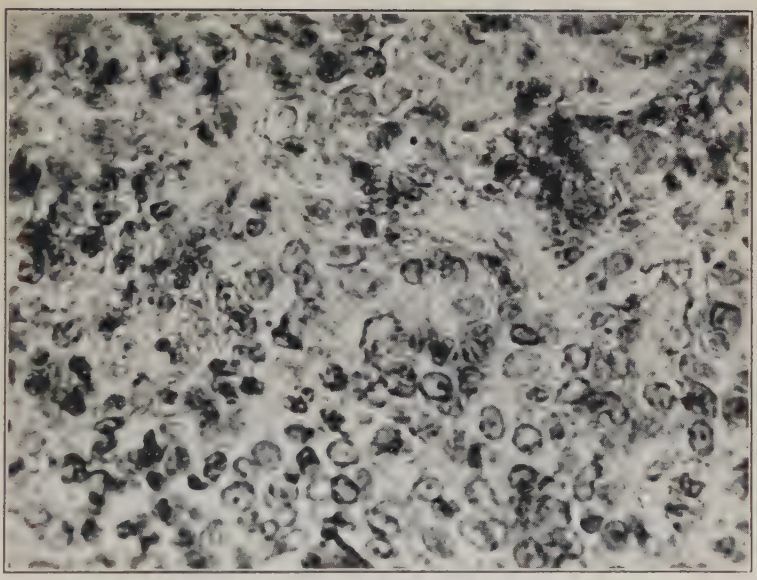


FIG. 26.—High-power field from the same preparation as Fig. 25. Taken near the larva, showing predominant histiocytic reaction (× 600). (From manuscript by Cannon and the author.)

(frequently called anaphylaxis) may be responsible for many of the symptoms encountered in parasitic infection. In addition to the references given, see Rachmanow (1913 and 1914), Paulian (1915 b) and Morenas (1922).

Finally, the question of the relation of hypersensitivity to symptoms should be systematically attacked from the pathological standpoint, such as has been done so fruitfully in tuberculosis (see page 216 and Krause, 1927). Cannon and the author (manuscript in preparation) are now carrying out just such a study with *Ascaris*, particularly in regard to artificially produced ascaris pneumonia in rabbits and guinea-pigs. The outstanding finding of this work is that the animals are sensitized during the first week of infection, so that, as the larvæ pass through the lungs (after the seventh day of infection), there is a tremendous allergic exudative reaction which gives rise to the pneumonia symptoms (Figures 25, 26).

V. *Summary*

In addition to the mechanical injuries produced by parasites, there are undoubted toxic manifestations produced by the absorption of toxic materials from the parasites. These materials may be classified as (1) true toxins; (2) other poisonous substances including endotoxins; and (3) proteins or protein products of the invader which are comparatively non-toxic to the normal host but become highly toxic to the host sensitized by infection.

The only undoubted case of toxin production by the parasites is sarcocystin from the Sarcosporidia. Sarcocystin stimulates the production of a true antitoxin and shows all of the classical characteristics of a true soluble exotoxin.

The exact functional rôle of the various poisonous materials, which are often termed endotoxins and are obtained from a large range of parasites, is uncertain. The clearest relationship is in malaria, where during the normal course of infection foreign proteins and various particulate materials are liberated directly and periodically into the blood-stream and where the symptoms may be anaphylactoid in character.

Parasitic infections often render the host hypersensitive to the proteins of the invader, as is evidenced by the various skin reactions described in Chapter V. The allergic exudative reaction of the host may account for the symptoms in *Ascaris* pneumonia. A special type of hypersensitiveness, i.e., anaphylaxis, may explain the symptoms following the improper removal of cattle grubs from the skin.

CHAPTER VII

THE CELLULAR BASIS OF IMMUNITY

I. *Introduction*

The usual distinction between the cellular and humoral reactions of the body is convenient, but purely arbitrary, because in final analysis both types of immunity have a cellular basis. Furthermore, it is becoming more and more apparent that all defense reactions, whether cellular or humoral (purely mechanical ones such as the protective action of the epithelium being omitted from consideration), are bound up with the connective tissue or "mesenchyme," which embryologically arises from the mesoderm.

Very little work has been done on this subject among the parasites and only a brief outline will be given in this book, as it belongs more properly to the province of pathology. In writing this chapter I have used as a basis Maximow's (1927) classical review of the "Morphology of the Mesenchymal Reactions." He recognizes three groups of cells of the connective tissue and blood:

1. The fibrocytes, which are fixed, highly differentiated cells and are represented by endothelial cells in the vascular system, by fibroblasts or fibrocytes in connective tissue, and by cartilage and bone cells in cartilage and bone. Some investigators would place the endothelium of the blood-vessels in category 2, but Maximow believes that this endothelium is so highly differentiated that its potentialities are limited to the formation of fibroblasts under certain conditions.

2. The fixed or free cells, usually termed histiocytes, which possess marked affinity for vital stains, are preëminently fitted for phagocytosis and the storing of various substances, and assume important rôles in the intermediate metabolism of the body and in defense reactions. These cells are known by a variety of names, such as macrophages, clasmatocytes, resting wandering cells, rhagiocrine cells and adventitial cells in the serous membranes and various irregularly arranged tissues; the reticular cells in lymphoid and myeloid tissues and the red pulp of the spleen; and the Kupffer cells in the liver. In addition there are modified flattened histiocytes lining the lymph

sinuses and venous sinusoids of the lymph nodes, bone marrow and spleen, which Maximow insists should be sharply differentiated from the true endothelium of the common blood-vessels belonging to the fibrocytes of category 1, and for which he suggests Siegmund's term, littoral cells (Uferzellen). This entire category of cells is the one which is included under the so-called reticulo-endothelial system.

3. The hemocytes, which are all free cells of the blood and lymph and accumulate in the blood-forming organs. They may be classified as follows: (a) Hemocytoblasts or undifferentiated stem cells from which the next types develop; (b) Differentiated cells: granulocytes (the eosinophilic, basophilic, and special or polymorphonuclear leucocytes), erythrocytes and megacaryocytes.

Before considering the actual work on the function of these various cells, specific mention should be made of the large monocytes in the blood because many authors consider that an increase of these cells (monocytosis) is evidence of a direct stimulation of the reticulo-endothelial system. The origin of the blood monocytes is a very controversial subject. Maximow believes that they are neither lymphoid nor myeloid, but that they represent further stages in the development of the lymphoid cells, lymphocytes or hemocytoblasts. He believes that a complete series of transitional forms can be found between the lymphocytes and monocytes, on the one hand, and between the monocytes and histiocytes, on the other. From this standpoint, a monocytosis is not a direct stimulation of the histiocytic system, but rather a stimulation to the formation of cells that can become histiocytes. As the monocytes have so many functions parallel to the histiocytes and can develop into them, it seems perfectly justifiable in the present review to consider them as a part of the reticulo-endothelial system.

The three groups of cells, the fibrocytes, histiocytes, and hemocytes, are all of importance in the defense of the body against parasitic invasion, but the brunt of the work, according to accumulating evidence, seems to fall on the histiocytes (reticulo-endothelial system). The part played by the various cells can best be indicated by considering their behavior in local and general defense reactions.

I. LOCAL DEFENSE REACTION

The local defense reaction (i.e., inflammation) varies with certain conditions and according to whether it is septic or sterile, but it is always fundamentally the same. It consists of three steps, each of which involves one of the three categories of cells. First, there is a

mobilization of the special leucocytes (polymorphonuclears, neutrophiles in man, microphages). These are true hemocytes which normally are limited to the lumen of the blood-vessels and to the myeloid tissue, but which in inflammation migrate through the walls of the blood-vessels and are the first to appear in the field of inflammation. If the inflammation is aseptic, they never become very numerous and soon disappear, but if it is suppurating they appear in enormous numbers. They combat the invaders by active phagocytosis and by the secretion of various bactericidal and detoxicating substances including proteolytic and oxidizing enzymes. These cells cannot multiply by cell division nor do they possess the potentiality to transform themselves into other types of cells. After their part in inflammation is over, a few may be taken away by the blood or lymph, but most degenerate and disappear *in situ*. Among the cells closely related to the polymorphonuclear leucocytes are the eosinophiles which frequently mobilize in the helminth infections. As will be shown later, present evidence indicates that they are important in the defense of the body against foreign proteins and function during various forms of hypersensitiveness.

The second phase of inflammation is a mobilization of the macrophages or mononuclear exudate cells. Pathologists are not in entire agreement as to the origin of these cells, but Maximow maintains that they have a dual origin from the local fixed cells, viz., from the histiocytes of the common loose tissue and serous membranes and from non-granular leucocytes of the blood. Compared with the special leucocytes which first appear in the field of inflammation, the macrophages have enormously greater potentialities for defense and repair. They actively phagocytize foreign bodies of all sizes and types, including protozoa, bacteria, dead cells and foreign particles; they liberate enzymatic substances, which are useful in ridding the inflamed area of various foreign materials, and when the inflammation subsides, instead of degenerating, they remain for the most part and take on the common structure of the normal histiocytes of the region or later become transformed into fibroblasts.

The third and final stage of inflammation is essentially regenerative and falls largely to the fibroblasts or "common" connective-tissue cells. These cells probably arise chiefly from preëxisting cells of the same type in the inflamed area and to a very much less extent from the endothelium of the small vessels. They form the new connective tissue with which the wound is repaired and may aid directly in defense by providing fibrous capsules to isolate foreign bodies or tenacious abscesses not cleared up by the macrophages.

Before leaving the question of local defense, mention should be made of the intense and very rapid inflammation seen in the skin reaction in the hypersensitive state. In regard to Arthus phenomenon, Maximow (1927) is of the opinion that the present rather scanty accumulation of facts indicates that "the differences, as compared with the histogenesis of common inflammation, are quantitative rather than qualitative. The obstruction of the blood-vessels of the affected area, the damaging of their walls and an intensive edema are the salient features. Increased degeneration of the fixed cells, on one hand, and increased migration of special and eosinophilic leucocytes and of lymphocytes, on the other, have been observed."

2. GENERAL DEFENSE REACTION

If the exciting cause (dissolved or particulate foreign material or infecting agent) is not localized, but is distributed over all or a large portion of the body, a general defense reaction occurs which, as some writers express it, follows upon an "activation of the mesenchyme." Such a general reaction involves all three categories of mesenchymal cells, just as did the local reaction, but their behavior is necessarily quantitatively different. Thus, for example, there is little need for the highly specialized fibroblasts.

Just as in the local reaction, one of the first signs of the general reaction is an increase in the special leucocytes (microphages) in the blood-stream (a leucocytosis). This is accomplished by an accelerated production of them in the bone marrow. In fact, toxic materials in the blood frequently stimulate leucocyte production in the bone marrow (although others deleteriously affect the bone marrow, causing a leucopenia). Such a leucocytosis, many pathologists feel, is not so much directed toward a phagocytosis of the invading microorganism as toward a resorption and detoxication of substances liberated by the invader. Parenthetically, it may be said that leucocytosis accompanies non-specific protein therapy. (See Petersen, 1922).

Whenever any substance is liberated in the blood-stream, it comes in contact immediately with the true endothelial cells of the blood-vessels, which Maximow classifies as fibrocytes. Of these, however, he says, "The rôle of the endothelium in the adsorption and neutralization of toxins and in other general reactions combating infections or intoxications may be important; but it is not manifest in appreciable morphologic changes."

The group of cells of paramount importance in the general defense reactions is undoubtedly the reticulo-endothelial system or the histio-

cytes. The way in which they remove various colloidal substances or suspensions that have been introduced parenterally is illustrated by their behavior toward certain dyes, India ink, etc. They are known to be important factors in the intermediate metabolism of various substances, such as fat, hemoglobin, etc. In addition rapidly accumulating evidence indicates that they are the chief elaborators of the humoral antibodies. Thus, on the one hand, organs particularly rich in reticulo-endothelial cells, such as, primarily, the spleen, and secondarily, the lymph nodes, bone marrow, liver, kidney, lung and omentum, seem to contain antibodies before the blood serum and, on the other hand,

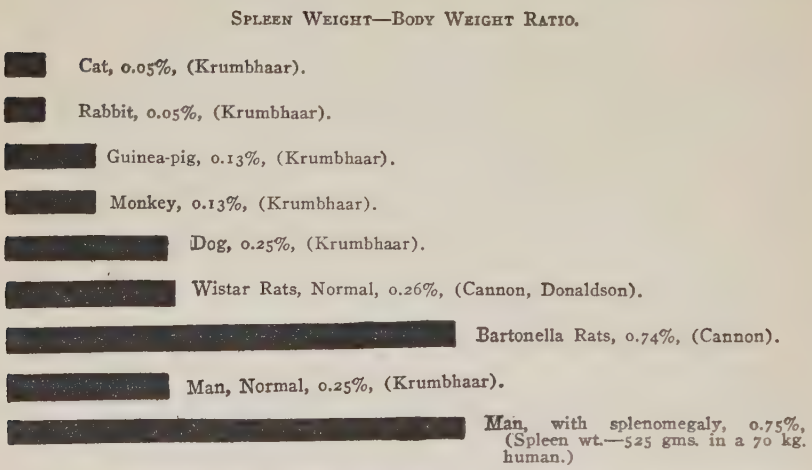

Spleen Weight—Body Weight Ratio.
Cat, 0.05%, (Krumbhaar).
Rabbit, 0.05%, (Krumbhaar).
Guinea-pig, 0.13%, (Krumbhaar).
Monkey, 0.13%, (Krumbhaar).
Dog, 0.25%, (Krumbhaar).
Wistar Rats, Normal, 0.26%, (Cannon, Donaldson).
Bartonella Rats, 0.74%, (Cannon).
Man, Normal, 0.25%, (Krumbhaar).
Man, with splenomegaly, 0.75%, (Spleen wt.—525 gms. in a 70 kg. human.)

Fig. 27.—Bar diagram showing the ratio of the percentage of spleen-weight to body-weight in man and certain laboratory animals. (From Cannon and McClelland, 1929.)

when deleteriously affected or blocked (as by injections with India ink) or extirpated, there is a concomitant decrease in antibody production. (See Howell, 1928.)

In all of this work on the site of antibody production, it must be borne in mind that the individual results are anything but clear-cut. On the whole, however, the variabilities seem to be more apparent than real. Inasmuch as splenectomy has an effect simply because the spleen is so rich in reticulo-endothelial cells, its removal in different mammals cannot be expected to have the same effect, because the relation of the size of the spleen to the body weight of the animal varies tremendously in different species, as may be seen in Figure

27. A general survey of the literature, as one would expect, then, indicates that the greatest effects on antibody production and resistance to infection are encountered in precisely those forms having the highest proportional spleen weight—rats and dogs. Furthermore, many negative results seem to have been due to unrealized technical difficulties. Thus, reticulo-endothelial destruction has to be tested within certain time limits, because the system is quickly regenerated by the proliferation of the cells in other localities such as the Kupffer cells of the liver. In blockading the reticulo-endothelial system in *Bartonella* infection of rats, Cannon and McClelland (1929) found that it was necessary to give enormous doses of India ink, whereas some investigators have used only single injections.

II. *Eosinophilia in Parasitic Infections*

The eosinophiles, according to the classification given in the introduction to this chapter, are true hemocytes and are closely related to the other granular leucocytes, such as the special leucocytes or microphages. In function, recent work has shown the two to be somewhat similar except that in the eosinophiles there is less emphasis on phagocytosis and more on the detoxication of foreign materials. In fact, the eosinophiles are supposed to be particularly concerned in the removal of foreign proteins or their split products. They, therefore, occur in great numbers in such conditions of protein hypersensitiveness as anaphylaxis, serum sickness and bronchial asthma, where they are found locally or generally, depending on the distribution of the foreign protein.

Eosinophilia is of particular interest in parasitology because it is so characteristic of infections of helminthic origin. In this field, an astoundingly large bibliography has accumulated, but most of it need not concern us here because it deals with such questions as the occurrence, extent and use in practical diagnosis. A few papers, however, deal with the mechanism of eosinophilia and the function of the eosinophiles.

In regard to the stimulus involved in the mobilization of the eosinophiles, a number of observers have been able to produce local or general eosinophilia by the injection of extracts of parasites or have been able to attract the leucocytes into capillary tubes containing extracts and placed in the tissues. (See the work of Vallillo, 1908; Panizza, 1910 and 1910 b; W. W. Herrick, 1913; Weinberg and Séguin, 1913, 1913 b and c, 1914, 1914 b and 1915.) Grosso (1912) was unable to check the finding of Vallillo and Panizza that extracts

of *Strongylus* were eosinophilotactic, but Weinberg and Séguin (1914), in view of their results showing that a local eosinophilia can occur only if there are eosinophiles in the blood, believed that Grosso's results were due to the use of animals with different quantities of eosinophiles in their blood. Most, if not all, eosinophilotactic mixtures contain proteins * and all available evidence indicates that they are most effective after a previous sensitization. The influence of previous sensitization is brought out by the work of Schlecht (1912) and Schlecht and Schwenker (1912) on various proteins and by the work of W. W. Herrick (1913) with extracts of *Ascaris*. (See also the work of Paulian, 1915.) Where extracts of parasites produce an eosinophilia without previous injection, the animals may have already become sensitized either to these or to some closely related parasite. Furthermore, Herrick found it impossible to produce an eosinophilia in guinea-pigs by injection with *Ascaris* extracts after the animals were immunized. Botteri (1925) has shown that one of the outstanding features of a twelve-hour positive intradermal test for hydatid is an intense local eosinophilia.

As to the stability of the eosinophilotactic substance, Homma (1921) reported that eosinophilotactic substances of parasites are not destroyed by the fixation and preservation of the parasites in formalin or alcohol and that they are both water and alcohol soluble. Paulian (1915) working with *Tænia, Oxyuris, Trichuris* and ascarids found them to be both alcohol and water soluble.

It has already been noted that an eosinophilia can be either focal or general. When focal, it occurs in the region of the fixation of the parasites, and the cells themselves are recruited from the circulating blood and arise in the bone marrow. Thus, Weinberg and Séguin (1913 b and 1914) were able to show that eosinophiles could not accumulate around the point of fixation in intestinal parasitic infections of horses unless they occurred in the circulating blood; that the number at the local site was proportional to the number in the blood and that the injection of certain parasitic extracts (eosinophilotactic substances) caused a temporary local increase of the eosinophiles at the site of injection and a concomitant decrease in the circulating blood. This last observation they have used to explain the curious fact that the eosinophiles may show an enormous decrease within a few hours after removal of hydatid cysts, since the spilling of any cyst fluid during removal would, by virtue of its eosinophilo-

* Even in such conditions as drug sensitivity (cf. Schlecht, 1912) where eosinophilia occurs, it may be brought about indirectly by proteins. See Boerner-Patzelt (1923) for a study of eosinophilia after the injection of enzymes.

tactic nature, result in an accumulation of the available eosinophiles at the point of operation with their consequent disappearance from the blood stream. Lemierre and Lantuejoul (1920) have explained in a somewhat similar fashion the post-therapeutic increase of eosinophiles which may persist for several weeks or even months following the removal of hookworms. Thus, the hypergenesis of the cells in the bone marrow cannot be discontinued as abruptly as is the eosinophilotactic stimulus; consequently, the cells accumulate in the blood.

As regards the general protective significance of eosinophilia, Milian (1901) found that the destruction of *Sarcocystis* in beef was associated with an intense leucocytic reaction in which the predominant type of cell was the eosinophile, and to a less extent, the mononuclear and mast cells.

Finally, special attention is due the work of Weinberg and Séguin (1915) on the function of the eosinophiles. They found that the eosinophilic leucocytes would phagocyte certain bacteria, fungi, protozoa, foreign red cells and inert particles, such as carbon, *in vitro* and *in vivo*. More particularly, they found that when eosinophiles were mixed with hydatid fluid and incubated one hour at 37° C. the cells lost their power to phagocyte certain organisms, and the cyst fluid lost its reactivity as a test antigen in complement fixation tests. This absorptive power was manifested to a less extent by the polymorphonuclears and was practically *nil* for the blood monocytes. In conclusion the authors remark, "Les leucocytes éosinophiles, comme les polynucléaires neutrophiles, jouent un rôle très important dans l'immunité. Mais alors que les neutrophiles ont pour fonction principale la protection de l'organisme contre les microbes, les éosinophiles sont surtout adaptés à la neutralisation de certaines substances toxiques. Les éosinophiles manifestent leur pouvoir phagocytaire en l'absence (totale ou relative) des polynucléaires neutrophiles. Il en est de même pour ces derniers qui peuvent se charger de l'absorption des produits vermineux en l'absence d'éosinophiles."

III. *The Reticulo-endothelial System in Parasitic Infections*

A general consideration of the preceding chapters indicates that as a rule the body reacts to invasions of parasites by the formation of antibodies and in some cases by obvious cellular changes. If the modern view of the predominant rôle of the reticulo-endothelial system in the body's defense against infection or the parenteral introduction of foreign material is correct, both of these responses should be traceable in large part to the reticulo-endothelial system. This is

becoming more and more apparent as interest is being aroused in the cellular responses of the body to parasitic invasion. So far, most of this work has been done with the protozoan infections. In the work on the reticulo-endothelial system, which has been ably reviewed by Linton * (1929), the following types of conditions can be recognized: (1) Infections, such as the leishmanioses, which are primarily infections of the reticulo-endothelial system; (2) Infections, such as malaria, where the chief defense of the body is probably a phagocytosis of the parasites by the reticulo-endothelial system; (3) Trypanosome infections where, in one case at least, a humoral antibody can be connected with the reticulo-endothelial system; (4) The curative action of certain drugs and normal human serum effective *in vivo* only by mediation of the reticulo-endothelial system.

I. LEISHMANIOSIS

Beginning with the work of Christophers (1904 and 1904 b) all of the pathological studies on kala-azar are consistent in recognizing this disease as primarily an infection of the reticulo-endothelial system of the body. Not only does the parasite live in the cells of this system but the proliferation of these cells constitutes the chief characteristic of the disease. Thus, Meleney (1925), after his study of the infection in man, monkey and the hamster, considered the two chief characteristics as (1) an extensive proliferation of the endothelial cells of the liver, spleen and bone marrow, and (2) the formation of large islands of clasmatocytes in the same organs. Confirmation of the localization of the parasites and the proliferation of the reticulo-endothelium may be found in the recent work of Perry (1922), Shortt (1923), Hu and Cash (1927), Cash and Hu (1927) and Pittaluga (1927). Several other points in these papers deserve emphasis. The last observer considered that the symptoms of kala-azar, such as anemia, leucopenia, enlargement of the spleen and liver, and hemorrhages in the skin, may all be explained by the fact that there is a blockade of the reticulo-endothelial system. Hu and Cash showed experimentally, by the injection of China ink into infected animals and by supra-vital staining of the cells obtained by spleen puncture from man, that the parasitized cells were actually clasmatocytes (histiocytes). And Linton pointed out that the parasitized

* Many of the statements made in this section are taken directly from Linton's manuscript, which he generously lent me before the final paper appeared in print.

cells, so carefully described by Shortt and Brahmachari (1925) in cases of dermal leishmanoid appearing in patients undergoing treatment for kala-azar, were undoubtedly tissue macrophages.

There are reports of *L. donovani* occurring in other than the reticulo-endothelial cells, but Linton believes that, "These occasional and in part unconfirmed findings do not weaken the main trend of the other observations, namely, that in kala-azar we are dealing with a disease whose primary attack is against the reticulo-endothelial system."

In kala-azar, then, the reticulo-endothelium seems to be successful in phagocyting the parasites, but the latter, instead of being digested, find the cytoplasm of the phagocytes a suitable medium in which to grow and multiply. Under such conditions, it is impossible to decide whether the reticulo-endothelium is valuable, as the only defense the body has, imperfect as it is, or deleterious, as being the most suitable location for the parasites. In this connection, a careful study of splenectomy during the disease in animals might be illuminating. It is true that splenectomy has been tried in man, but the results are too variable and uncertain to draw definite conclusions. Thus, in 1910 Alvares successfully splenectomized a child with kala-azar without, however, arresting the disease. Jemma and di Cristina (1911) believed that such a procedure could not be expected to be beneficial, since the parasites occur in other organs beside the spleen, and that cases apparently cured were probably not due to *Leishmania.* Makkas (1911), on the other hand, contended that it could be of value if performed during the early stages of the disease when the spleen is almost invariably the only organ affected; he gave as evidence two kala-azar cases (one of which died of an intercurrent pneumonia) which showed improvement following the operation at seven and nine months' illness, respectively. A similar improvement was noted in one case by Melissinos (see Makkas, 1911), in one out of three cases (the other two died) by Kokoris (1915), and in two out of three cases by Cochran (1915). In dogs infected with *L. donovani,* according to Nicolle and Comte and Laveran (cited by Laveran, 1917), splenectomy did not arrest the progress of the disease, and Laveran (1917), in his book on the leishmanioses, seemed to feel that splenectomy in kala-azar was of doubtful value.

Dermal leishmaniosis also seems to be characterized by the parasitism and proliferation of the clasmatocytes. As Linton points out, the original report of Riehl (1886) and the classical description by Wright (1903) show these cells to be affected.

The genus *Leishmania* is not unique in parasitizing the reticulo-endothelium of vertebrates. A number of the sporozoa behave similarly. Thus, in the malaria-like *Hepatozoön canis,* schizogony occurs in the mononuclear cells of the spleen and bone marrow and the gametocytes in the circulating mononuclears. (See Christophers, 1906; and Wenyon, 1911.) The same may be true of *H. muris* of the rat (Wenyon, 1926) and of the *Hepatozoön* of the Indian palm squirrel (Patton, 1906).

As has been pointed out for kala-azar, it is very desirable that any or all of these parasites be studied from the standpoint of the course of infection, resistance, and antibody production in relation to splenectomy and reticulo-endothelial blockade. The data so far are very incomplete. As indicated in Chapter II, the antibody production is very low in cases of kala-azar, is higher in persons spontaneously cured, and is still higher in the more self-limited dermal leishmanioses. Furthermore, the highest titers are obtained in animals injected with leishmanial products, where they do not become infected. As far as it goes, this evidence would seem to indicate that the invasion of the reticulo-endothelial system in kala-azar does not stimulate it to a greatly increased antibody production, but rather effectively blockades the system.

2. MALARIA

The study of phagocytosis of the malarial parasites is handicapped by the apparent rapidity with which the parasite is digested, and the relationships established so far are chiefly based on the distribution of malarial pigment within the body. This, however, is not an infallible criterion since a great deal of the pigment is obviously phagocyted as pigment (after disintegration of the parasites or after sporulation) and since as yet it is not certain that all the cells which will take up pigment will ingest parasites or the parasite red cell combination.

Pathological studies of fatal cases of malaria indicate that the pigment is found stored chiefly in the reticulo-endothelial system. Only a few of the many papers will be mentioned. R. H. Jaffé (1927), in reviewing the rôle of the reticulo-endothelial system in pathologic conditions in man, pointed out that in patients dying of malaria the malaria pigment is most distinct in the reticulo-endothelial cells of the hematopoietic organs and produces a picture resembling vitally stained animals. Some observers (see Dudgeon and Clarke, 1917; Gaskell and Millar, 1920; and Seyfarth, 1926) have also described the pigment as being taken up by the capillary endothelium of other

organs, especially the brain. This again raises the question as to whether or not the capillary endothelium forms a part of the true reticulo-endothelial system. In his own work on severe estivo-autumnal malaria, however, Jaffé (1927) found that most of the pigment in the capillaries of the brain was enclosed in free, large, mononuclear cells and in enlarged adventitial cells, but was only attached to (not engulfed by) the swollen and fatty degenerated endothelial cells. Phagocytosis of *P. falciparum* was described by Gaskell and Millar (1920) as being carried on in the spleen by the "branched supporting cells" and in the liver by the stellate cells. Linton identifies the former as the reticulum cell and the latter as the Kupffer cell, both of which are histiocytes. Wenyon (1926) pictured the phagocytosis of *P. falciparum* as seen in a spleen smear from a fatal case. A monocytosis is frequently found in the blood in malaria (Lucey, 1921; Meo-Columbo, 1924; Naegeli, 1925; and Ottander, 1926) and may possibly be evidence of a stimulation of the reticulo-endothelial system. Some authors (D. Thomson, 1911; S. P. James, 1920; V. Schilling, 1924; Anderson, 1927) lay particular stress on the occurrence in the blood of the large mononuclears which have been repeatedly described as containing pigment (Naegeli, 1925; Seyfarth, 1926) and which, according to V. Schilling (1924), are identical with the proliferating monocytes of the spleen. Infected red cells tended to adhere to them in cultures of *P. falciparum,* according to McLay (1922).

Experimental work on avian malaria has led to much the same conclusion as has that on human malaria, namely, that the chief defense against malaria is phagocytosis by the reticulo-endothelial system. Thus, MacCallum (1898) observed phagocytosis of bird malarial parasites and Ben Harel (1923) concluded that the chief destruction of the parasites was by the fixed tissue phagocytes, particularly of the spleen. She based her conclusions on the following types of evidence: the presence of pigment in the histiocytes of the spleen, the accumulation of parasites around the detached monocytes in the spleen, and the increase of mononuclears in the blood paralleling the increase of parasites. Indirect evidence to the same effect has been given by an extensive series of experiments carried out by the author and L. G. Taliaferro (1929, 1929 b and see page 137). Briefly, the basic fact was observed that if enormous numbers of washed parasitized cells are introduced intravenously into (1) a bird with a latent infection, and (2) a normal bird, they will be removed from the blood of a latent bird, sometimes within a few hours, whereas in a normal bird they immediately initiate an acute infection which fre-

quently proves fatal. (See Figure 18.) In attempting to find the mechanism of such an immunity, however, no endothelial opsonins, lysins or antibodies with protective or curative properties have been demonstrated. These results, in conjunction with the former work, indicate that the method of defense is one of phagocytosis, without the mediation of humoral antibodies.

Recently, Ruge (1927) has suggested that the constant presence of malarial pigment within the reticulo-endothelial system prevents the formation of anti-malarial antibodies and explains why there is no immunity in the disease. This suggestion can hardly be taken seriously when it is recalled what enormous doses of India ink, for example, are necessary to block the reticulo-endothelial system of experimental animals (see page 227), and when it is pointed out that according to R. Jaffé "at least 10 c.c. of colloidal silver per kilogram are required to obtain something approximating a temporary blockade of all the histiocytes," whereas small amounts will only stimulate the cells.

For one reason or another the spleens of a number of persons infected with malaria have been removed, but the results are too conflicting to warrant very definite conclusions in regard to the effect of the operation on the infection. The advisability of splenectomy in malaria is a mooted question. In many of the cases that have been reported, observations on the operation were made from the viewpoint that the spleen had become so dangerously enlarged that rupture was feared, and consequently relapse of the malaria was not considered or not considered sufficiently. Improvement after splenectomy, with no record of relapse, has been reported by Rosati (1912), Cumston (1919) and Raison (1920). Minervini (1915) felt that the operation might be successful when the spleen had become degenerative, provided other hematopoietic organs had had time to take over the function of the spleen. Degorce (1913), on the other hand, believed that extirpation of the spleen interfered with the defensive mechanism of the organism and that before the operation, if any parasites were found in the peripheral blood, energetic quinine treatment should be administered. In line with this, Strine (1914) and Sidgwick (1925) reported intense relapse in a case after splenectomy. Others have obtained variable results. Kopylow (1913) in a historical review found that the mortality (not necessarily relapses, however) ranged from 7 to 60 per cent, and of his own thirteen cases, relapses occurred in three. Cartolari (1913) stated that relapses may or may not occur. Gonder and Rodenwaldt (1910) noted that relapses of *P. kochi* in apes followed splenectomy and that infections were much

more severe in splenectomized animals than in unsplenectomized animals. Noguchi (1928) also recorded a relapse of malaria in a rhesus monkey following splenectomy.

3. PIROPLASMOSIS

There is considerable evidence that splenectomy lowers the body's resistance to piroplasm infection. Gonder and Rodenwaldt (1910) produced a relapse in a dog with a latent infection of *Babesia canis* by removal of the spleen. Working on the same parasite, Ciuca (1912) found that splenectomy produced no effect on the infection provided the dog had completely recovered from the operation before infection, but seemed to produce an effect when performed during the course of the infection. In the latter case, the author pointed out that shock or accidental infection attendant upon the operation rather than absence of the spleen might be the causal factor. In spite of the justice of this hesitation to ascribe the effects to splenectomy, it should be pointed out that the fully recovered animals probably had made good a large amount of the reticulo-endothelial system, lost by splenectomy, by proliferation in other sites of the body. Working with a piroplasm of the ape, Kikuth (1927) found that unsplenectomized animals recovered, but splenectomized animals succumbed. Regendanz and Kikuth (1928) produced a relapse of an unknown and unsuspected piroplasm infection in four out of six Brazilian opossums that they splenectomized.

De Kock and Quinlan (1926) found that *Gonderia* (= *Theileria?*) appeared in the blood of sheep after splenectomy although they had not been observed before. They produced no untoward symptoms and could not be transmitted by blood inoculations to other animals (either splenectomized or unsplenectomized). In a more extended study, de Kock and Quinlan (1926 b) reported that after splenectomy relapses followed with severe symptoms in horses infected with *Nuttalia* (= *Babesia*), in bovines infected with *Babesia bigemina* and *Theileria mutans* and in sheep infected with anaplasms, and with milder symptoms in bovines infected with anaplasms; and that after splenectomy no important sequelæ developed in animals which did not harbor piroplasms or anaplasms or both, but that their subsequent injection with parasites produced a decidedly more acute and prolonged course of disease. The blood changes in the splenectomized infected sheep were associated with a marked oligocythemia and the appearance in the blood of erythroblasts, normoblasts and Jolly bodies. Moreover, first a neutrophilia was noted (probably due

to the operation), then a monocytosis with erythrophagocytosis, a lymphocytosis and occasionally an eosinophilia. (See also de Kock, 1928.)

4. TRYPANOSOMIASIS

The clearest-cut case of the rôle of the reticulo-endothelial system in the trypanosome infections is in the formation of the humoral antibody, described by the present author (1924) in *T. lewisi* infections, which inhibits the cell-division of the parasites, but which does not kill them (page 125 and the supplementary work of Coventry, 1925, and others on page 127). Regendanz and Kikuth (1927) verified the formation of this reaction product and have further shown that in splenectomized rats division of the parasites continued several days longer than in non-splenectomized controls and that in some splenectomized animals the trypanosomes never ceased to divide so that the infection behaved like a pathogenic one and terminated fatally. The findings of Regendanz and Kikuth have been confirmed in the main by the present author, Cannon and Goodloe (unpublished data). In a series of experiments, we found very little effect if splenectomy was performed before injection. If, however, it was carried out at certain times after the rat had already formed the reaction product, it resulted in a "relapse of reproduction," that is, the adult trypanosomes began to divide again, just as they did during the acute stage of the infection, and went through a typical cycle of reproduction which ceased in about ten days. Ordinarily no further relapse of reproduction took place, but in one case a second relapse occurred during gestation. The fact that splenectomized rats again form the reaction product is probably due to a regeneration of the reticulo-endothelial system in the liver, as is indicated by the great increase in Kupffer cells following operation. The efficacy of splenectomy in producing these relapses of reproduction was dependent on a number of conditions: Splenectomy was most effective during those parts of the infection when, according to the previous work of Coventry (1925), the titer of the antibody was lowest: in other words, when there was a large amount of the antibody in the blood, the rat had a margin of safety until proliferation of the system in other parts of the body permitted the formation of sufficient quantities of the antibody. Splenectomy could be made effective during any part of the infection if combined with India ink blockade. Likewise, it was more effective when there was a concomitant infection with *Bartonella,* which probably causes a partial blockade of the reticulo-endothelial system.

Various investigations have indicated that phagocytosis of the trypanosomes by the leucocytes of the blood (chiefly the large mononuclears) and the fixed tissue phagocytes is an important factor in the resistance of the host. (See pages 130-133.) Undoubtedly phagocytosis occurs under some conditions, although it has never been observed by the present author or by certain other investigators in *T. lewisi* infections, but it seems open to question as to whether it may not be simply a clearing up of parasites made moribund by humoral antibodies. Under such conditions, it would represent a necessary, but subsidiary process, and its activity, comparatively speaking, would depend upon the titer of the humoral antibody. Thus, when the titer is high, the parasites are lysed quickly before the mononuclear leucocytes ingest them, but when low, the parasites are lysed slowly, and in the meantime are ingested by the phagocytes.

With the demonstration of a relation between the spleen and the formation of the reproduction-inhibiting reaction product in *T. lewisi* infections, it becomes of interest to study its relation to the formation of lysins. Some evidence on this subject exists in studies made on the comparative length of life of normal and splenectomized animals that have been infected with various species of pathogenic trypanosomes, but the results are very conflicting. In reviewing this work it is interesting to note the explanations given for the results. It must be remembered, however, that the earlier authors were interested in the rôle of the spleen and other organs *per se* and not as a part of the general collection of cells now known as the reticulo-endothelial system. Thus, Bradford and Plimmer (1902) found that rabbits, dogs, cats and rats splenectomized before infection with *T. brucei* died more quickly than controls, a fact which they considered bound up with phagocytic activity. On the other hand, in the same year, Laveran and Mesnil (1902 c) found that the course of a nagana infection in a splenectomized rat was not different from the control. Later, Sauerbeck (1905) confirmed Bradford and Plimmer's work and further found upon examination of organs at autopsy that trypanosomes underwent more rapid alteration in the spleen than in other organs, notably the liver. Rodet and Vallet (1906 b) also ascribed remarkable trypanolytic properties to the spleen which they maintained was trypanolytic *in vitro* as well as *in vivo*. Following this work, Roux and Lacomme (1906) treated three dogs, infected with nagana, with splenic extract of normal cattle. This caused a temporary decrease of parasites; but as pointed out by Rodet and Vallet (1906 c), such temporary decreases may have been due simply to trypanolytic crises. After an extensive in-

vestigation Laveran and Thiroux (1907 and 1907 b), on the other hand, could find no difference in the course of infection with *T. evansi* in two splenectomized rats and five guinea-pigs as compared with controls, could find no heightened phagocytic action in the spleen nor any trypanolytic action of extracts *in vitro,* and, furthermore, contended that the altered appearance of trypanosomes in spleen smears was due to the difficulty of staining them in dried tissue smears, since a little fluid containing some blood, when drawn from the spleen and stained, showed trypanosomes as numerous and as normal as elsewhere. Finally, they concluded that the rôle of the spleen was limited to removing the debris from the circulation after trypanolytic crises. In further experiments, however, Rodet and Vallet (1907 and 1907 b) maintained that in *T. brucei* there is actually a destruction in the spleen (an extracellular lysis) and that the property developed in rats without a crisis gradually, but in guinea-pigs developed at the first crisis and persisted until death. In the same year, Massaglia (1907 b) repeated the work of both sets of investigators and finally confirmed the work of Laveran and Thiroux by concluding that the spleen had no special trypanolytic property. Gottberg (1908) concluded similarly. Later, Lanfranchi (1910) reaffirmed the trypanolytic property of the spleen because he found that by inoculating trypanosomes into the spleen, the resulting infection proceeded at a slower rate. Parenthetically it may be mentioned as indicative of the desires held at the time but not realized later, that Lanfranchi expressed the hope that animals might be immunized with such an attenuated virus.

Mutermilch (1911) performed *in vitro* lytic experiments using *T. brucei* and various normal and infected organs and blood, extracted in saline for three or four hours at 38° C., and then centrifuged. When obtained from an infected guinea-pig before the crisis, extracts of liver, bone marrow and, preëminently, spleen lysed the trypanosomes, whereas extracts of kidney, serum and leucocytes did not. After the crisis, all the extracts (except leucocytes) were equally effective. Extracts from normal animals were ineffective. From these and other experiments, he concluded that in guinea-pigs infected with *T. brucei* the trypanolytic antibodies seemed to be formed in the hematopoietic organs, but were rapidly poured into the blood, and when depleted by bleeding were made good by the cellular tissues. Rondoni and Goretti (1913) in a study of guinea-pigs, rats and mice infected with *T. brucei* also concluded that the spleen possessed a certain degree of trypanolytic power.

Concomitantly with this work, pathological observations showed

that among the most common anatomical alterations in trypanosomiasis is enlargement of the spleen. The hypertrophy of the spleen, according to Laveran (1908), is sometimes enormous (the spleen represented one fifth of the body weight of an infected mouse, as compared with one three-hundredth in the normal), but varies with the species of host and duration of infection. It appeared more marked in the mouse, rat, guinea-pig and dog which die with more or less large numbers of parasites in their blood than in the rabbit, goat, sheep and cattle in which parasites are only rarely found. It was more marked in acute than in chronic infections.

Curiously enough, while some workers were trying to endow the spleen with destructive properties, others were supposing it to be a refuge during trypanolytic crises. The question arose, where were the trypanosomes during the crises? According to Salvin-Moore and Breinl (1907), the parasites took on latent forms (latent bodies) in the spleen which were capable of developing later, at opportune times; and according to Fantham (1910), they developed in the lungs and congregated in the spleen and bone marrow as little more than non-flagellated forms, possessing a nucleus and parabasal body, which were infective when injected into normal rats. Corroborative evidence by Fusco (1910) tended to show that blood from an infected animal which was passing through a trypanolytic crisis due to an arsenical was not infective to fresh animals, whereas fragments of spleen were. All this evidence was later thrown out of court, however, when Hindle (1910), in studying degenerating forms of *T. gambiense* in rats treated with arsenophenylglycin, found that the nuclei were the last parts of the parasites to disappear and that it was they that were probably interpreted as latent bodies. Laveran (1911 e) came to the same conclusion. Finally, Laveran and Mesnil (1912), in their excellent review, summed up the situation by saying that resistant forms did not need to be postulated, in view of the fact that ordinary forms could be demonstrated in the host's blood during trypanolytic crises by subinoculation methods, provided enormous amounts of blood were employed, even though they could not be demonstrated by direct microscopical examination of fresh or stained blood films.

Recently Linton (1929) could find no effect of splenectomy on the length of life in guinea-pigs infected with *T. equiperdum*. Similarly the author, T. L. Johnson and Cannon (unpublished work) could find no effect on mice infected with *T. equinum*.

The sum total of this work on the effect of splenectomy in infections with the pathogenic trypanosomes seems very conflicting. In-

herently, though, the various conditions, as indicated by accumulating work, were not conducive to clear-cut results as the following facts show: (1) Rats and mice are the animals best suited for this work from the standpoint of their high spleen weight: body weight ratio. On the other hand, these animals are least acceptable for this work from the standpoint of the infection since only very occasionally in the case of the rat do they form trypanolysins or show any marked acquired resistance to the pathogenic trypanosomes. (2) Among the larger animals studied (see figure 27), guinea-pigs and rabbits exhibit infections proceeding by trypanolytic crises and relapses which conceivably might be altered, but the spleen weight: body weight ratio is so low that it is quite possible that splenectomy does not produce a quantitatively sufficient disturbance of the reticulo-endothelium. The situation is further complicated by the fact that the length of life of the animals is unpredictably variable. Further studies on dogs, however, might be very profitable.

I have already noted that Kligler and Weitzman (1926) found that they could produce relapses in rabbits infected with *T. evansi* by the injection of olive-oil, and that as they could demonstrate no humoral antibodies, they believed the resistance to be cellular in character, and temporarily broken down by a blockade of the reticulo-endothelial system. Since, however, I cannot find that they tested for humoral antibodies on passage strains kept in mice (see page 121), this work does not seem to me to furnish evidence for the purely cellular basis of the resistance, but rather for the cellular basis of humoral antibodies.

Finally, there is a growing mass of evidence that the reticulo-endothelial system performs an important rôle in the activation of certain non-immunological trypanocidal agents, particularly normal human serum and drugs. I have already made a detailed comparison of the trypanocidal property of normal human serum and immune serums. In view of the fact that repeated doses of human serum rendered an animal incapable of being further protected by human serum and that this could not be explained on the basis of the formation of an anti-substance because the protective property was not antigenic in mice or rabbits, Rosenthal and Freund (1923) were led to suggest that human serum was not trypanocidal *per se* but was activated by some mechanism in the body—that it was not trypanocidal but trypanocidogenic. Thus, repeated doses rendered an animal non-susceptible to the protective property because the activating mechanism was exhausted. Later work by Rosenthal and Spitzer (1924) indicated that this activating mechanism was located in the

reticulo-endothelial system. Thus, mice treated with thorium-X and almost completely deprived of circulating leucocytes still activated human serum, but blockade of the reticulo-endothelial system with saccharated iron oxide sometimes prevented activation, splenectomy caused a pronounced lowering of activation, and combined blockade and splenectomy invariably made the mice incapable of activating the human serum and resulted in a fatal infection. Furthermore, they showed that saccharated iron oxide did not act directly on human serum to render it incapable of being activated.

Much the same rôle of activation by the reticulo-endothelial system is being brought out by some of the recent studies on the chemotherapeutic activity of certain drugs. A large number of references can be found in the literature indicating that a given drug is active *in vivo* in much lower concentrations than *in vitro*. It is from these discrepancies that various authors have proposed the hypothesis that certain drugs induce the production of unknown metabolites by the body (see, for example, the work of Lipkin, 1919, on malaria) or of parasiticidal antibodies by the body (see, for example, Schilling and Jaffé, 1909, on trypanosomes; and Yorke and Macfie, 1924, on malaria). Linton pointed out that an analysis of the discrepancies between *in vivo* and *in vitro* activity of drugs from a cellular standpoint was begun almost simultaneously by Jungeblut (1927), Feldt and Schott (1927) and Kritschewsky and Meersohn (1926) who studied the effect of splenectomy or blockade of the reticulo-endothelial system with saccharated iron oxide or India ink or both in drug-treated infections of *T. brucei,* a spirochæte and a streptococcus, respectively. In all cases the efficacy of a given drug was greatly lessened by splenectomy or blockade. Essentially the same results were reported by Kritschewsky (1927) and Kolpikow (1926) who tested the efficacy of drugs at different intervals after splenectomy and found that the longer after splenectomy the drug was given, the less the effect. In other words, there seemed to be a replacement of the system, as has been indicated in other studies.

IV. *Summary*

Investigations on the cellular basis of immunity in the parasitic infections have centered around the function of the eosinophiles, the direct phagocytic activity of certain cells, and the rôle of certain cells in the production of humoral antibodies or the activation of certain parasiticidal mechanisms.

Eosinophilia, either general or local, is characteristic of the hel-

minth infections. In both cases, the eosinophiles arise from the bone marrow, and the localized accumulations are recruited from the general circulation. Although the eosinophiles exhibit many activities similar to the polymorpholeucocytes, such as phagocytosis, their predominant rôle seems to be defense against foreign proteins. Eosinophilotactic substances have been obtained from many worms and their efficacy in producing eosinophilia is largely dependent upon previous sensitization.

The reticulo-endothelial system itself is known to be parasitized by the *Leishmania* and certain of the sporozoa, which find it a suitable medium in which to grow and proliferate, and by their invasion, appear to inhibit or greatly decrease antibody production; it is the chief defense of the body in malarial infections; it is responsible for the formation of the humoral antibody which inhibits cell-division of *T. lewisi,* and it is important in the activation of the trypanocidal property of normal human serum and drugs.

CHAPTER VIII

THE NATURE AND PRODUCTION OF IMMUNITY IN PARASITIC INFECTIONS

I. *Introduction*

Although such terms as immunity and host's resistance are extremely useful, they are always, as has been pointed out in Chapter I, relative. Thus, a host may be immune to a parasite, but by subjecting the host to conditions which lower its resistance, or by subjecting the parasite to conditions which increase its virulence or by disturbing the quantitative relations, as by giving an abnormally large infecting dose of parasites, a seemingly complete immunity may be broken down.

Just as antibodies and various immune processes are classified as natural or acquired, so immunity is classified. A natural immunity is one exhibited by a species or individual which is not dependent upon past infection or upon past contact with derivatives of the parasite, but is an expression of the fundamental biological incompatibility between a given host and parasite. An acquired immunity is the direct result of infection or some immunizing procedure, whether past or present. As defined, this includes the so-called immunity to superinfection.* Some authors (cf. Blacklock and Gordon, 1927) seem inclined to restrict the term to those cases in which the exciting infection has completely disappeared from the host, but this seems an impossible distinction because in many bacterial and protozoan diseases it is a purely "academic" question whether the immunity is actually a sterile one or whether it is an immunity to superinfection.

Of particular interest is the occurrence of age immunity which may be defined as a change (most frequently an increase) in resistance with advancing age. It is frequently loosely used to designate the existence of a lower infection rate in high age groups as com-

* Immunity to superinfection is known under a number of names, for example: tolerance (Plehn), infectious immunity (Kolle), dumb infections (Reiter), labile infections (C. Schilling) and premunition (Ed. Sergent).

pared to lower ones. In some cases, as in diphtheria immunity, it is generally held to result from past subclinical infections. In other cases, it probably represents a self-limited infection together with greater exposure to infection among the lower age groups. In still other cases, as in certain worm infections, it can be shown experimentally to parallel the aging process *per se* and not to be the result of infection. In its strict sense and as used in the present discussion, however, the term is restricted to cases of natural resistance without relation to previous infection.

II. *Natural Immunity*

Natural immunity is an expression of host-parasite specificity. As was noted in Chapter I, no host has either a universal immunity or a universal susceptibility to all parasites. Furthermore, some parasites are very host-specific and can live in only one or a few hosts under normal conditions, whereas others can live in a large number of hosts. Between these extremes every gradation is found.

Although in some cases, as far as experimental results have shown, a given natural immunity may be absolute, in many cases it is a relative matter depending on such matters as dosage, virulence of the parasite, physiological condition of the host, etc. The effect of dosage may be illustrated by the large amounts of *Leishmania donovani* necessary to infect dogs. The effect of virulence may be illustrated by the high natural immunity of mice to *Trypanosoma lewisi,* which Roudsky (1910, 1911 b and 1911 c) was able to overcome by an exaltation of the virulence, involving large doses and rapid passage, so that the parasites not only infected mice, but became pathogenic to them. The effect of the physiological state may be illustrated by the increased susceptibility following vitamin A deficiency in chickens to *Ascaridia* (Ackert, Fisher and Zimmerman, 1927) and in pigs to *Ascaris* (Hiraishi, 1926, and Koidzumi, 1927). Furthermore, a parasite may be very host-specific although it may infect a large range of hosts in which it only partially completes its life-history. Thus, *Ascaris lumbricoides* of the pig will infect rats, mice, guinea-pigs and rabbits, but will only go through the first part of its life-cycle in them and never reaches sexual maturity in the intestine.

Very few cases of natural immunity to the parasitic diseases have been studied adequately enough to ascertain the bases for the im-

munity. In many cases the immunity seems due to one or several non-specific factors of the host's body, such as the thickness of the skin or the action of digestive juices, etc. Thus, Andrews (1927), in a study of the coccidia, found that in the normal host the digestive process facilitated excystation of the oöcysts, whereas in an abnormal host it allowed the oöcysts to pass unchanged through the intestine. Other factors were also of importance, because he found that when injected intraduodenally, certain merozoites produced infection in their normal host, but did not in any other host. Other examples of this type are probably furnished by the condition of the marmot which cannot be infected with the trypanosomes, *T. gambiense* and *T. evansi,* during hibernation at 6° C., but can be during active life (Blanchard and Blatin, 1907), or the similar condition in the dormouse (Brumpt, 1908) where the animals infected with *T. gambiense* generally recovered when hibernation was induced. Brumpt believed that the trypanosomes (*T. gambiense*) disappeared because their vitality was so lowered that they were readily taken up by the phagocytes which retained their vitality, whereas the natural trypanosome of the animal (*T. blanchardi*) could survive in the blood during hibernation. Such cases seem to be associated with temperature as well as various physiological conditions of the host.

In some cases the body fluids may possess actual parasiticidal properties. For example, Laveran and Pettit (1909) divided the cold-blooded vertebrates into three groups in respect to their natural immunity to *T. evansi:* (1) those, such as *Tropidonotus natrix,* which were very receptive; (2) those which allowed only a few trypanosomes to pass from the peritoneal cavity into the blood where they persisted only a short time; and (3) those, such as *Triton vulgaris, Rana esculenta, Anguilla vulgaris,* in whose blood the parasites never appeared. Of the latter group, they worked with the eels. Trypanosomes, upon addition to eel's serum, rapidly lost their motility and were lysed, as could be seen in hanging-drop preparations. Members of the second group possessed this trypanolytic property to a less extent. Massaglia (1909 b) likewise attributed the destruction of *T. evansi* in the peritoneal cavity, blood and lymph of adders, tortoises, frogs and lizards to a trypanolysis and could find no indication of phagocytosis. In the early study of the same property in rat serum against *T. paddæ* of birds, Levaditi and Sevin (1905 b) found that it was destroyed at 56° C. and could not be reactivated by either normal mouse or guinea-pig serum. It seems, therefore, to be different from the immune trypanolysins (page 119). Laveran and

Pettit similarly found that the property was destroyed at 58° C. In the examples just given the serum of the naturally immune host has a direct parasiticidal property and can undoubtedly function in the immunity. In some cases, however, investigators have fallen into the error of ascribing a natural immunity to an indirect parasiticidal property of the serum. Thus, as discussed on pages 167-168, the natural immunity of man to the animal trypanosomes was correlated with the trypanocidal activity of human serum *in vivo;* but later work demonstrated that human serum was not trypanocidal *per se,* but stimulated the production of a trypanocidal metabolite in the animal (mouse) in which it was tested.

In many cases the body phagocytes seem to play an important rôle in the natural defense of the body. Among the numerous examples that could be cited are the extensive investigations of Delanöe (1911 and 1912) on the natural immunity of mice against cultural forms of *Leishmania* and various trypanosomes. He concluded that the immunity is mainly of a phagocytic nature. A similar investigation was carried out by Visentini (1912 and 1912 b) on the natural immunity of guinea-pigs and rats to cultures of *Leishmania donovani* following intraperitoneal injection. They ascertained that within ten minutes the flagellates adhered to the leucocytes and were soon ingested by them. Scorda (1912) confirmed these results in guinea-pigs and rabbits, but found that *in vitro* digestion of the flagellates was very slow, ingested forms being still recognizable after twenty-four hours, whereas *in vivo* they were destroyed in two hours. In work of this nature, it is difficult to distinguish between phagocytosis and parasiticidal mechanisms. Undoubtedly, in some cases, primary phagocytosis is predominantly important, but in others, it may be subsidiary in that the phagocytes may only ingest parasites which have already been devitalized by other mechanisms. This is suggested, for example, in the work of Brumpt, cited above.

Many cases of natural immunity, especially when the immune animal is far removed systematically from the normal host, may simply be due to a lack of suitable nutritional materials for the parasite. This is essentially Ehrlich's atreptic immunity and seems a reasonable assumption although little or no experimental proof has, as yet, been advanced to support it.

The natural immunity to true toxins (compare work on sarcocystin, page 203) is often due to a lack of affinity between the body cells and the toxin (in Ehrlich's terminology, a lack of receptors). In such cases not only is the host uninjured, but antitoxin is not produced.

III. *Age Immunity*

As pointed out before, true age immunity does not result from past or present infection or contact with derivatives of the parasite. It is, therefore, a natural resistance not dependent on any specific mechanism in the immunological sense. Sandground (1929), in his review of the increase in resistance with age to helminth infections, found that it was, as a rule, characteristic only of *abnormal* hosts of the parasite, and made the interesting suggestion that the adults of these abnormal hosts are so specific in certain physiological characters as to be unsuitable for the parasite's development, while the young are less specifically differentiated, so that the parasites can gain a temporary foothold. In other words, among several species, the young would resemble each other more than the adults, and, as a consequence, a given parasite, normally adapted to one host, would live in the young of several other hosts but would find it increasingly difficult with advancing age. No hypothesis has been advanced to explain the reciprocal condition of decrease in resistance with age, such as in the *Babesia* infections of cattle where infection in young animals is less severe than in adults.

Many examples of age immunity might be cited, but as most of the careful work in this line has been done recently with the helminth infections, the present discussion will be limited to them. Parenthetically, it may be pointed out that in many cases of so-called age resistance, based on statistical studies, such questions as the influence of previous infection, changing habits and consequent lessened contact with infection, etc., have not been adequately eliminated.

An age resistance of dogs to *Ancylostoma duodenale* was reported by Looss (1911). Young puppies tolerated the growth of the worms, but older dogs were refractory to infection. This problem has been studied in detail by C. A. Herrick (1928) in *A. caninum* in the dog and cat, and by Sarles (1928) in *A. braziliense*.

The studies of Ransom (1921) indicate a similar condition (Table 29) in chickens toward the "gapeworm," *Syngamus trachea;* this condition does not exist in its normal host, the turkey. *Ascaris* in pigs, according to the same author (1922), probably belongs in the same category.

Particular consideration is due the work of C. A. Herrick (1925) on the nematode, *Ascaridia lineata,* of the chicken. Herrick was able to demonstrate conclusively, in chickens raised under controlled conditions, that age resistance was not connected with past infection. Furthermore, he studied its rate of development by feeding chickens

TABLE 29

RESULTS OF ARTIFICIALLY INFECTING CHICKENS WITH THE GAPEWORM, SYNGAMUS TRACHEA (FROM RANSOM, 1921)

Age of chickens when fed	*Number fed*	*Number infected*	*Per cent infected*
1-4 weeks	47	41	87
5-8 weeks	32	27	84
9-20 weeks	32	21	66
21 weeks to adult	28	8	29

of different ages with nematode eggs and ten days later autopsying them to determine the incidence of infection and the average length of the parasites. The length the parasites attained in ten days gave a direct measure of the suitability of the host for the parasite. A graphic representation of Herrick's results is given in Figure 28, from

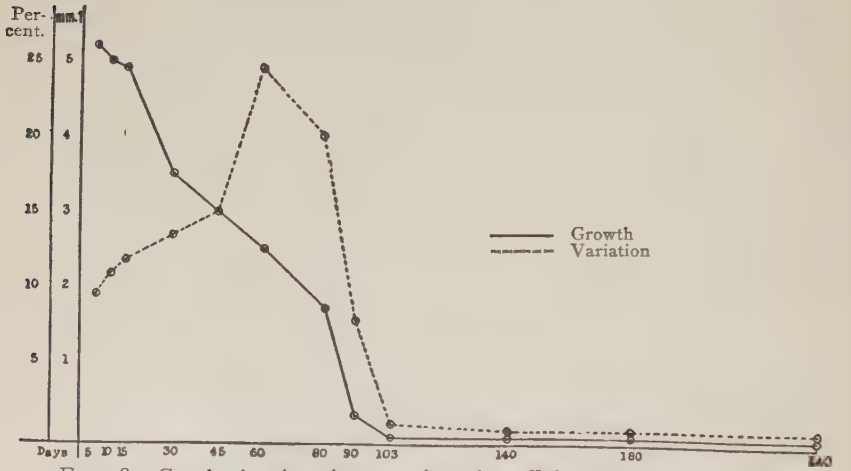


FIG. 28.—Graph showing the growth and coefficient of variation of *Ascaridia lineata* developing in chickens of different ages. (After C. A. Herrick, 1925.)

which it can be seen that in five-day-old chicks, the worms actually grew over 5 mm., that they grew progressively less as the chicken grew older until in 103-day-old chicks, the newly hatched larvæ were practically unable to grow (their gain in length was 0.1 mm.). In attempting to transfer this age resistance passively by injecting young chicks with serum from older ones, undoubtedly positive results were obtained with the serum of one cockerel. The first test with the serum

was its administration curatively into a 22-day chick possessing a ten-day infection, with the result of an undoubted vermicidal action; a second sample was tried protectively on three chicks in 1, 2 and 3 c.c. doses, with the result that the chick receiving the largest dose harbored no parasites, although untreated controls harbored over 2,000 each; a third sample strengthened the evidence of strong protective action; but subsequent samples were without effect. Less clear-cut results were obtained with the serums of seventeen other chickens, but Herrick feels that their serums made the chickens more resistant to the effects of the parasites, although the parasites themselves were not killed. In trying to modify the age resistance of the chickens, thymectomy and the prevention of the flow of bile into the intestine did not affect the growth of the parasites, but removal of large amounts of blood by cardiac puncture made the chickens less able to cope with the growth of the parasites.

IV. *Acquired Immunity in Infections with Protozoan Parasites*

It is neither practicable nor desirable to list all of the reported or alleged cases of acquired immunity developed by innumerable hosts against their equally innumerable parasites. Instead, I shall deal with certain well-recognized cases where experimental studies have been directed toward the elucidation of the mechanism involved and the production of artificial immunization.

In considering the various methods which have been used to produce immunity artificially in animals, it is necessary to differentiate between active and passive immunization. In the first, either the parasites (dead or alive), their secretions, or their disintegration products are introduced into the experimental animal, and the animal then produces its own antibodies. In the second, ready-made antibodies which have been formed in another animal are introduced into the experimental animal. The various methods of producing immunity have been outlined on page 14. Suffice it to say here that practically all the successful results in the artificial production of immunity in the parasitic infections have been attained by active immunization.

1. LEISHMANIOSIS

Of the leishmania infections particular interest is due oriental sore (caused by *L. tropica*), because in many instances primary infection confers an immunity to a second sore. This was known long before the etiological agent was discovered and in some places was

applied practically by inoculating material from a sore on an unexposed portion of the body to prevent the occurrence of a sore with its scar on the face. The organism can be transmitted to dogs, monkeys, mice, rats, etc. The inoculation is generally fatal to mice but reinfection of the few that recover occurs so that the mice evidently acquire no immunity whatever (Laveran, 1915). In experimentally inoculated dogs, Nicolle and Manceaux (1910 and 1911) found that upon reinoculation when incompletely or recently cured, a condition of sensitization resulted in a shortened incubation period, but when completely cured an immunity sometimes occurred. Laveran (1916) confirmed and amplified this work. One of his experiments will illustrate the type and duration of immunity found. A first inoculation into a dog of *L. tropica* (from mice) resulted in a sore containing many parasites, which lasted ninety-five days; two months after complete cure, upon a second inoculation, a sore also with many parasites lasted but thirty-eight days; a third and fourth inoculation were negative; upon a fifth inoculation (109 days after cure of the second infection), a small sore with very few parasites lasted forty-eight days; a month later, upon a sixth inoculation, a pronounced sore with a moderate number of parasites lasted forty-three days; upon a seventh and eighth inoculation, sores were again produced. Monkeys, on the other hand, acquired a more rapid immunity than dogs. At times one inoculation prevented reinfection, but not invariably (Laveran 1917 b and 1918). Although most humans acquire an immunity as a result of a single infection, there are exceptions recorded in the literature. (See, for example, Bouilliez, 1918.) Marzinowsky and Schourenkoff (1924) found that an experimental sore confers immunity only when it is allowed to run a natural course.

As far as I am aware, the attempts to produce immunity to *L. tropica* with vaccines have not been successful (cf. Parrot, Donatien and Lestoquard, 1927); this is in line with the findings of Marzinowsky and Schourenkoff that abortive sores confer no immunity. Row (1912) has, however, given some preliminary experiments in which he believed a vaccine of *L. tropica* was helpful in hastening cure. In kala-azar, according to Nicolle (1910), Nicolle and Manceaux (1910), Nicolle and Comte (1910 and 1911) and Nicolle and Anderson (1925), a first attack produced an immunity in dogs and monkeys (if not fatal), provided the cure was complete and of several months' standing; before that time, it sensitized the animals. Nicolle and Manceaux (1910) showed that after cure of kala-azar a dog was immune to oriental sore, but that a monkey cured of oriental sore was not immune (or only partially so) to kala-azar. Laveran (1914)

showed that a monkey inoculated with the Mediterranean strain of kala-azar was refractory to the Indian strain.

In common with oriental sore, attempts to treat kala-azar with vaccine have been unsatisfactory. Killed cultures were used by Longo (1912) on two cases and by di Cristina and Caronia (1912) on seven cases.

2. TRYPANOSOMIASIS

1. *Occurrence of acquired immunity.* The clearest-cut example of a successful acquired immunity in the trypanosome infections is in the non-pathogenic *T. lewisi* in the rat. Kanthack, Durham and Blandford (1898) first established the fact, which has been repeatedly confirmed since, that a rat recovered from an infection with *T. lewisi* is refractory to a second inoculation. Laveran and Mesnil (1901) came to the conclusion that the immunity and the final removal of the parasites are due to the phagocytosis of living trypanosomes. MacNeal (1904), on the other hand, held that the crisis and subsequent immunity are due to a trypanolysin, just as has been described for the pathogenic forms. Manteufel (1909) agreed with this view. The author (1924) and Coventry (1925 and unpublished work) found that a passively transferable trypanocidal property, probably a lysin, is formed which terminates the infection and that a reproduction-inhibiting property (page 125) is also developed. These two factors together seem to be an adequate basis for the immunity to reinfection, with phagocytosis possibly playing a subsidiary rôle. For example, a rat recovered from an infection of *T. lewisi* receives a few parasites from an infected flea. These find their way eventually into the blood-stream, but on account of the reproduction-inhibiting property are unable to reproduce. Moreover, they are relatively so scarce that microscopic examination fails to reveal them. Hence, they simply exist there for a time and are unable to set up an infection. The lysin eventually sweeps them away. In the recent work of Regendanz and Kikuth (1927), the hypothesis was advanced that there is no lysin formed, but that after reproduction is inhibited, various non-specific agencies, such as phagocytosis by the reticulo-endothelial system, continuously remove the adult trypanosomes. In view of the work of Coventry previously cited, I cannot agree with them in this belief.

The so-called pathogenic trypanosomes are generally lethal to most domestic and laboratory animals. Sheep and goats are notable exceptions and their infections have been described by Laveran (1911 and 1911 f) and others. The infection is of an extremely

chronic and extended type, but recovery takes place (four to twenty-one months), and thereafter an immunity exists which sometimes lasts as long as twenty-eight months (against *T. evansi*). The immunity is specific. In fact during a period of several years, Laveran successively infected a goat with *T. pecaudi* (= *T. brucei*), *evansi* and *gambiense* and a sheep with *T. pecaudi* (= *T. brucei*), *dimorphon* (= *congolense* in part) and *T. congolense*. Little is known of the mechanism. Furthermore, the infections are difficult to study because of the scarcity of parasites. It is true that the serums of recovered sheep and goats develop a protective property (see Chapter IV) which Laveran (1911) and Mesnil and Leger (1912) believed could be used as an index of an animal's immunity. For example, when the serum of a recovered animal is injected simultaneously with the original passage strain, it prevents infection. In consideration of certain facts, however, this property cannot unreservedly be considered to account for the immunity. In the first place, the serum does not protect against trypanosomes taken from the host during infection. And, in the second place, the property is developed even in laboratory animals where the trypanosomes eventually prove fatal.

Most of the work up to the present on immunity to superinfection has been carried out on infections in mice treated with drugs and indicates that the immunity is extremely specific. In this respect it resembles the resistance of trypanosomes to antibodies (page 118). In 1925 Kritschewsky and Awtonomow found that mice, after infection with a salvarsan-resistant strain of *T. brucei* and treatment with salvarsan, remained infected and could generally be superinfected with related and identical races of *T. brucei*. Brussin and Rubinstein (1925) confirmed this work in the main, but pointed out that Kritschewsky and Awtonomow did not actually use identical strains and that when such identical relapse variants were used superinfection did not take place. Such an immunity was sharply race-specific, just as after cure (page 284), and did not extend to different relapse variants. It lasted from twenty days to two months. In discussing immunity to superinfection, Brussin and Rubinstein believed that there was no justification for assuming an "immunitas non sterilisans," dependent upon the presence of living organisms in the body, and, therefore, separate and distinct from other types of immunity. Kritschewsky and Heronimus (1927), working with *T. brucei* and *T. equiperdum*, carried out additional experiments and confirmed the previously obtained results. In all of this work, the identity or non-identity of the various races and variants were ascertained by the Rieckenberg blood-platelet test (page 120).

2. *Artificial production of acquired immunity.* Investigators have attempted to secure active immunization against trypanosome infections by three methods:

1. Infections with a living but attenuated virus.
2. Infections with a virulent virus followed by treatment with drugs.
3. Injection of a killed virus.

Although this work, especially the last two methods, has led to some extremely interesting results with laboratory animals, it has not been used successfully with man or domesticated animals.

IMMUNIZATION BY INFECTION WITH A LIVING BUT ATTENUATED VIRUS

A large number of investigators have attempted to immunize animals by infecting them with attenuated strains of trypanosomes, but although the length of life of the infected animals may be considerably increased, death follows just as surely, so that practically no success has, up to now, been attained. Some representative samples may be cited.

Koch (1901) and Schilling (1902, 1904 and 1905) made use of the attenuation following passage through certain animals, to immunize cattle against *T. brucei.* The former used a strain passed through a rat and a dog, whereas the latter used a strain passed several times through either rats or dogs (in some of his work through eighteen to twenty-one dogs). These were seemingly successful, but as Laveran and Mesnil (1912) pointed out, the results may have been explained by the fact that cattle frequently recover spontaneously from nagana. Martini (1905), after inoculating two donkeys with *T. togolense* (= *T. brucei*), originally gotten from a stallion and passed through white mice, found that the donkeys resisted, for a long time, inoculation of a virus which was invariably fatal for untreated donkeys; but his immunized animals finally died and their blood was itself infective to other animals (see Kleine and Möllers, 1906). Even Koch (1904) gave up his original procedure because he recognized the danger of spreading the disease by means of virulent organisms in his so-called immunized animals. Sporadic experiments continued, however. Offermann (1913) injected a mare intravaginally four times with blood from rats and mice infected with *T. equiperdum;* but although the mare showed no pronounced symptoms and no trypanosomes microscopically, her blood was infective to mice during febrile

periods while agglutination and complement fixation tests were positive.

Darling (1913) seems to have been more successful in his work with *T. hippicum.* He reported that a strain which had survived 336 days in a guinea-pig had lost much of its pathogenicity, for upon inoculation into a dog and mule, both became infected, but recovered. Later, this and other mules were inoculated with a virulent strain and treated with an arsenical, but only the recovered mule survived. Somewhat similarly, another mule, after recovery from the avirulent strain, was refractory to the virulent one. It is quite possible, of course, that real recovery after infection with attenuated strains is easier with *T. hippicum* than with *T. gambiense, T. evansi,* etc. Of interest in this connection is the fact that *T. equinum,* which is closely related to *T. hippicum,* sometimes shows spontaneous recovery in infections in guinea-pigs.

Working with *T. lewisi,* Novy, Perkins and Chambers (1912) reported the successful immunization of rats with attenuated cultures, which were started in 1905, were non-infective to rats even in large doses and could not be restored to infectivity at the 158th subculture, and at the time of the paper were in the 313th subculture. Active immunity developed in rats receiving one or multiple doses of the living, non-infective cultural trypanosomes. It arose in about ten days and lasted for life. Sometimes very large doses of organisms produced short abortive infections. The authors hoped to apply their method to the pathogenic forms with which encouraging results had already been obtained in the case of *T. brucei.*

Rondoni and Goretti (1913) treated isolated *T. brucei* with distilled water, a hypertonic solution of saline, salts of quinine or salvarsan. Upon the injection into mice, the vaccine, prepared by the first three methods, caused a definite prolongation of the incubation period (lived five to twelve days longer than controls) when virulent organisms were inoculated a week or more thereafter, whereas the vaccine prepared by the fourth method resulted in eight apparently complete immunizations (trypanosomes not found within twenty to twenty-five days) and a prolongation of the incubation period in seven (three to seventeen days). The serum of rabbits treated with the salvarsan vaccine, when injected simultaneously with trypanosomes, protected mice somewhat (four to ten days). This vaccine was prepared by placing isolated trypanosomes in contact with salvarsan (1:40,000) for thirty minutes at room temperature, then recovering them by centrifugation. These results, however, could not be repeated by Laveran and Marullaz (1914).

INFECTIONS WITH A VIRULENT VIRUS FOLLOWED BY TREATMENT WITH DRUGS

Mention has already been made of the early work of Ehrlich and Shiga (1904), Halberstädter (1905) and Franke (1905) showing that if a mouse be cured of an infection with a pathogenic trypanosome it is refractory for about twenty days to another infection. These general results were confirmed and greatly amplified by Browning (1908), Terry (1909 and 1909 b) and Schilling (1909), although the length of the immunity depended on the drug strain of trypanosomes, number of trypanosomes at the time of treatment, size of infecting dose and other factors. (See page 283 for this and other work in relation to species differentiation, and page 284 in relation to strain differentiation.)

Certain workers have also used animals other than mice and rats. Thus, the Sergents and Lhéritier (1919) have described an immunity in a dromedary after infection with *T. berberum* (= *T. evansi*) and treatment; in this case reinoculation produced a low grade transient infection followed by recovery. Kleine and Fischer (1922) worked on monkeys infected with *T. brucei* which when cured with Bayer 205 developed a more persistent immunity than when given a prophylactic dose of the drug alone. This has been confirmed by Kligler and Weitzman (1924, 1926 and 1926 b) with *T. evansi* in rabbits and guinea-pigs.

Although of great theoretical interest this method of immunization has two great drawbacks for practical application. In the first place, it cannot be used on man or valuable animals until a practically perfect method of treatment is evolved. In the second place, it is so specific that immunity to a given strain will not protect against antibody variants of the strain.

IMMUNIZATION WITH KILLED TRYPANOSOMES

The earlier attempts to immunize laboratory animals with dead trypanosomes or their disintegration products were almost all either entirely negative or simply prolonged the life of the immunized animal a short time over that of the control. Of those who were unable to influence the course of a subsequent infection, Laveran and Mesnil (1902) used *T. brucei* which had been rendered uninfective by being kept on ice for twenty-four hours, heated to 40° C. for several hours, or treated with dyestuffs; Mayer (1905) used saline extracts of washed, isolated and concentrated *T. brucei* and *T. equinum*, and Uhlenhuth and Woithe (1908) killed their organisms (*T. equiper-*

dum), which they obtained from infected organs and blood, by drying, by mechanical and chemical means and by heating to 55-60° C.

Working with the non-pathogenic *T. lewisi,* Novy (1907) was the first to report the successful immunization of rats. These he gave three or more injections at two-day intervals of cultural trypanosomes which were plasmolysed with distilled water and dialysed through collodion sacs. Later, Manteufel (1909) obtained negative results when the *T. lewisi* were heated to 60° C. for one hour or dried at 37° C., but partial immunity when the organisms were treated with distilled water or drugs. His criterion of immunity, however, was based on the number of organisms in the blood of the subsequent infection which, in view of the wide range encountered in normal infections, does not seem to the writer a valid measure unless a very extensive series be studied. A certain lengthening of life in immunized animals was reported by Ross and J. G. Thomson (1910). They found that one out of three rats lived longer than controls when treated with a saline suspension of a "vaccine" prepared by heating a saline suspension of *T. rhodesiense* to 55° C. for half an hour and adding tricresol. Similarly, Latapie (1911) lengthened the life of five mice to fourteen days and of one mouse to thirteen days, whereas four controls lived only ten days. Laveran (1911 d), on the other hand, found that mice which were injected with powdered *T. gambiense* and subsequently with virulent organisms, died much sooner than the controls. This result he ascribed to trypanotoxins contained in the powder.

Some of the more recent investigations have given much more promising results, although they have sometimes not been confirmed. Schilling (1912; for a general review, see Schilling, 1927), having noticed in his work of 1909 that there was an immunity following the cure of *T. brucei* infections by means of arsenophenylglycin, attempted in 1912 to produce an immunity in animals by the injection of trypanosomes which had been killed *in vitro* with tartar emetic (two hours' contact of 1:700 dilution). Following the injection of 0.5-2.0 c.c. of the killed isolated trypanosomes, an undoubted protection occurred in rats and mice which were inoculated six to ten days later with virulent organisms. Furthermore, 1 c.c. doses of serum from these immune rats mixed with the parasites protected mice. Two horses immunized with this material remained trypanosome-free four weeks after injection of *T. brucei* and showed protective properties in their blood. In a dog, after a single injection of the antigen, antibodies were demonstrated both by protective experiments with mice and by complement fixation. Laveran (1912 d) met with little success

in repeating Schilling's results with *T. brucei.* A transitory immunity was, however, obtained in mice with *T. rhodesiense.*

Probably the most thorough investigation in this field is to be found in the series of papers by Teichmann and Braun (1911) and Braun and Teichmann (1912, 1912 b and c). The last mentioned paper contains a valuable and detailed summary of all of their work. These authors used, as a source of their antigen, washed isolated trypanosomes, dried at room temperature, which, for immunization, were suspended in saline and a little toluol added. Some of their more important findings may be noted. Of 118 mice immunized with five doses of 0.02 gm. each of *T. equiperdum* powder, 98 per cent exhibited protection when inoculated seven days after the last injection of antigen; when eighty of these were reinfected three to four weeks later, 78 per cent remained immune; and when sixteen of these were, for the second time, reinfected three months later, six remained immune; of ten rabbits immunized with the same organism, two animals receiving two doses of 0.05 gm. of powder were not protected against a subsequent infection, whereas the remainder receiving from three to five doses were; and of eight rats and eight guinea-pigs, the first immunized with five doses and the second with four doses of 0.04 gm. of *T. brucei* vaccine, six animals in each lot were protected against an injection of virulent *T. brucei* seven days after the last dose of antigen. There was no specificity between *T. equinum, T. brucei* and *T. equiperdum.* Thus, mice immunized with one were protected against the others, but were not protected against *T. gambiense.* These group reactions are all the more interesting in view of the fact that a sharp specificity was obtained between passage strains of a given species and a serum-fast variant, as will be pointed out later. In trying to confirm Braun and Teichmann's work, Laveran and Roudsky (1913) prepared the *T. brucei* antigen in a like manner except that they used aseptic precautions and did not add toluol, but the treated mice upon infection only survived controls by one to three days. In fact, unlike Braun and Teichmann, they found the dried trypanosome material toxic to mice. Aoki and Kodama (1913) were likewise unsuccessful in treating rabbits, rats and mice with dried *T. equiperdum.* This they attributed as probably due to a wait of twelve days after the last dose of vaccine to test the immunity, instead of the five-day wait which Braun and Teichmann used. Similarly, Hintze (1915) reported practically negative results with dried *T. brucei* in rats, guinea-pigs and rabbits and also with spleen pulp and liver from infected animals and serum from guinea-pigs and rabbits following a crisis. As the author himself remarks,

however, immunity might have been obtained with larger doses of vaccine, but these he avoided as being prohibitive in any practical application.

Schilling and Rondoni (1913) found that an efficient immunizing antigen could be prepared from *T. brucei* by suspending in physiological saline and heating at 37° C. for varying lengths of time (four and a quarter hours was probably best). Mice given doses of 0.04 to 0.25 c.c. of such material showed varying degrees of resistance when injected seven days later with virulent organisms. More recently, Ponselle (1923), with a vaccine prepared from the blood (about 0.5 c.c.) of a mouse in the terminal stages of *T. brucei,* mixed with 2.5 c.c. of a phosphate buffer solution (pH 6-7.5) and left for twenty to twenty-four hours at 20° C., obtained marked protection in 70 per cent of mice (numbers not given) receiving one dose of 0.1 c.c. and eight to ten days later injected with virulent organisms, while thirty per cent died four to five days after the controls.

A very interesting series of observations has recently been published by Kligler and Weitzman (1926 and 1926 b), in which the length of the parasitological incubation period was used as an index of the resistance of the host. They found that injections of autolysed (freezing and thawing) or heat-killed (54° C.) parasites (*T. evansi*) rendered rabbits hypersensitive instead of immune. Thus, two rabbits, each given five sets of five injections at two-day intervals, showed parasites in their blood the first day following injection as contrasted with the fifth day in the control. Laveran's (1911 d) findings, which have been already reviewed, may thus be accounted for. Parenthetically it may be said that these results do not necessarily contradict the positive immunization experiments of other investigators because hypersensitiveness is probably one phase of the immunity process, that is, the phenomenon of resistance or of hypersensitiveness will be called forth depending upon the technique and time intervals employed. In contrast to the hypersensitiveness evidenced in rabbits treated with trypanosomal materials alone, Kligler and Weitzman found a marked increase in resistance following the injection of rabbits with parasites suspended in a dilution of Bayer 205, which of itself was insufficient to explain the results. The length of the incubation period in four rabbits was fourteen, nineteen and twenty-one days and no infection as compared with six, eleven and ten days with drug alone, and seven, four and five days with control infections. The rabbit which showed no infection was subsequently reinfected on the forty-sixth day.

Taken as a whole the results which have just been reviewed are

very conflicting. Undoubtedly under certain conditions very promising results can be obtained, as shown by such investigations as those of Braun and Teichmann. The question arises as to why so many investigators have failed to repeat these findings, using essentially the same technique. It seems to me that these failures probably depend on certain difficulties that are hard to control:

1. There is the fact, recognized by Schilling (1914) and others, that different strains of trypanosomes (even of the same species) differ in their antigenic power to produce immunity.

2. Various resistant strains may play a part. From the discussion already given on page 121, of the origin of serum-resistant strains, it is obvious that when investigators carry their passage strains in other animals than mice (or rats for some trypanosomes) the strain used for testing the immunity of the experimental animal may be a relapse strain of the one used as the source of vaccine. It is true that the lack of specificity between *T. brucei, T. equiperdum* and *T. equinum,* established by Braun and Teichmann, rather indicates that such fine differences between antibody-resistant strains of the same species do not matter; but on the other hand, some of their results may be interpreted otherwise. Thus, of ten mice immunized with five doses of 0.02 gm. each of a vaccine prepared from *T. equiperdum* (Strain 1), five were protected against the same strain, but the other five were not protected against *T. equiperdum* (Strain W), and conversely, when the mice were immunized with Strain W vaccine. (Strain W was obtained by subjecting Strain 1 to homologous anti-rabbit serum in mice until it became antibody resistant.) Such specificity did not, however, occur in all strains. Thus, when mice were immunized with Strain sf (a second variant of the serum-fast Strain W) vaccine, there was protection against Strains sf and 1, but not against Strain W. This variability in the antigenic behavior of different variants from the original strain need not be emphasized here, but rather the fact that sometimes the acquisition of antibody resistance is accompanied by a change in antigenic properties such that animals successfully immunized with one vaccine may not be immune to infection with one of these variants. This makes it imperative not only to keep the vaccine strain in mice (see page 121), but also to test it at frequent intervals to see that no change has taken place.

3. The particular technique or time of testing may find the animal in a hypersensitive state. That such may occur is indicated by the work of Kligler and Weitzman and may explain the results of Laveran. As has already been pointed out, this hypersensitiveness,

although giving the opposite effect to immunization, is probably a phase of the same process.

4. In all work on immunization great care should be taken to standardize the infecting dose of virus with which the immunized animals are tested. There is every reason to believe that all immunity is relative so that a given animal may be immune to a certain dose of virulent virus and yet have this resistance completely overcome by a large dose, as the work of Novy, Perkins and Chambers (1912) on *T. lewisi* indicates.

3. PIROPLASMOSIS

The piroplasms are parasites of the blood corpuscles of mammals which differ from the malarial parasites in that they do not form pigment. As some difference of opinion exists as to their valid names, it seems worth while to list the more important species and the diseases which they produce. In doing this, I have followed the nomenclature of Wenyon (1926). *Babesia bigemina* produces the disease of cattle known under a variety of names, such as Texas fever, red water fever; *B. bovis,* a similar hemoglobinuric fever in cattle; *B. mutans,* a benign fever of cattle; *B. motasi, B. ovis,* and *B. sergenti,* diseases of sheep corresponding, in order, to the three babesias of cattle; *B. caballi* and *B. equi,* hemoglobinuric fevers in horses; *B. canis* and *B. vitalii,* malignant jaundices in dogs; *Theileria parva,* a serious disease of cattle, known as East Coast fever, which differs from red water fever in that hemoglobinuria, jaundice, and progressive anemia are absent; and *Theileria hirci,* a somewhat similar disease in sheep. Resulting from these infections, there seem to be three typical immunological conditions: the benign infection with *B. mutans* gives no immunity to superinfection (see Ed. Sergent *et al.,* 1924 and 1927); in the pathogenic *Babesia* infections, a long period of latent or low grade infection is associated with a high resistance to a second attack of the disease; and in *Theileria* infections, a complete recovery (generally) is followed by a high resistance to subsequent attacks.

Among some of the pathogenic infections, remarkable success in immunization has been attained. As attention has been centered mainly on the infections of *Babesia* and *Theileria* in cattle, where, as a consequence, most of the practical applications have been made, these will be used as representative examples in the following discussion. For a review of the work in the production of immunity to the *Babesia* infections of the horse, sheep and dog, the reader is referred

to the review of Velu (1922). Although a great deal more has to be done, the underlying principles are similar to those in *B. bigemina.*

1. *Babesia.* For many years it had been common knowledge in this country that if cattle were brought from the northern to the southern United States, they contracted Texas fever (one of the names expressive of this condition is "acclimation fever") and frequently died. Investigation later showed clearly that the southern cattle escaped because they were already innocuously infected. According to Salmon and Smith (1905), "The earliest accounts we have of this disease date back to 1814, when it was stated by Dr. James Mease, before the Philadelphia Society for Promoting Agriculture, that the cattle from a certain district in South Carolina so certainly disease all others with which they mix in their progress to the north that they are prohibited by the people of Virginia from passing through the state; that these cattle infect others while they themselves are in perfect health, and that cattle from Europe or the interior taken to the vicinity of the sea are attacked by a disease that generally proves fatal."

Investigators soon recognized that one attack of Texas fever conferred an immunity to later infections (Smith and Kilborne, 1893; Koch, 1898; M. Nicolle and Abil-Bey, 1899; and Tidswell, 1900). They also early demonstrated that the immunity was not associated with a complete riddance of the infection, but with a persistent low-grade infection. Schroeder and Cotton (1907) noted a case in which the infection persisted twelve years. Concomitantly many of them clearly realized the dangers of relapse, either spontaneously or due to intercurrent infection, environmental action or injection of foreign materials, which was not, however, so likely as in malaria (Smith and Kilborne, 1893; Pound, 1895; M. Nicolle and Abil-Bey, 1899; Lignières, 1900; Carpano, 1914).

Experimental immunization is essentially a reduplication of the conditions in the southern cattle and consists in setting up a low-grade or latent infection. Because of its practical application and extensive use, it is not possible to review all of the contributions or to consider them historically. An excellent résumé of the early work is given by Kossel (1902) and of the more recent work by Velu (1922) in his monographic treatment of the piroplasms. Immunizing procedures (some of them of only historical interest now) have ranged from: infection by ticks (100 or more), themselves either naturally or experimentally infected (Dawson, 1902; see also Connoway and Francis, 1900); infection by ticks experimentally infected on recently immunized cattle (Dodson, see Dalrymple, Dodson and

Morgan, 1899); injection of macerated infected ticks; injection of blood preserved at 5-8° C. (Lignières, 1903); injection of blood from cattle in various stages of the disease, particularly after recovery from the natural attack; and infection followed by treatment with drugs.

The expedient of injecting blood from infected cows after recovery from acute symptoms was suggested by Smith, Kilborne and Schroeder (1893), and was tried at about the same time in several countries by Schroeder (1899, who started work in 1895), Dalrymple (see Dalrymple, Dodson and Morgan, 1899) and Connoway and Francis (1900) in America; by Koch (1898) in Africa; and by Pound (1897 and 1899), Tidswell (1900) and Hunt (1899) in Australia. This has developed into the method *par excellence* and consists in injecting a small amount (1-10 c.c.) of blood from an animal that has recovered from a natural or an induced attack of the disease. Practical applications followed rapidly and may be found in papers by Cary (1901), Francis (1902), Kossel (1902), Kossel, Weber, Schütz and Miessner (1903), F. Chambers and Smith (1914), Misson (1914), Bevan (1919), Velu (1922), Ed. Sergent *et al.* (1927) and many others. As representative of the results attained, Nesom, Shealy and Foster (1904) stated that of the non-immunized cattle imported to the tick-infested regions of the Southern United States, 50 to 90 per cent died, whereas of 4,750 immunized cattle only 7.6 per cent died, either from inoculation or subsequent naturally contracted fever.

The greatest subsequent advance was the treatment of artificially contracted infections with trypan blue whose action on the piroplasms was discovered by Nuttall and Hadwen (1909) and is known as the Theiler method. Its distinguishing feature is that it allows immunization to be applied to very susceptible animals because severe symptoms or unexpected flare-ups can be controlled. The animals are not cured, but their infection is reduced to a low grade, thereby insuring against superinfection. Treatment is usually started after the appearance of parasites in the blood (for some recent work, see Ed. Sergent *et al.*, 1927). Not only is it applicable against *B. bigemina,* but also against *B. canis, B. caballi* and *B. motasi.* It is not generally considered effective against the other species of *Babesia* and *Theileria,* but Yakimoff (1926) reported that it acts on *B. bovis,* and he and his collaborators (1928) have used it in immunization.

As immunization against Texas fever consists in producing the disease by inoculating virulent virus in order to make use of the active immunity to superinfection which develops during the

long continued latent infection, the question naturally arises as to how this can be done without the inevitable high mortality evidenced when uninfected cattle are introduced into Texas fever regions.

Undoubtedly, the first and foremost answer to this question is the fact, recognized by all the early investigators (see for example Smith and Kilborne, 1893), that there is a well-marked age resistance (the reverse of the usual condition) which is evidenced by an *increased severity* of the disease *with age*. The younger the calf, the more chance for a successful outcome. A second matter of importance, within certain limits, is the size of the infecting dose. Although some authors have contended that it was immaterial whether the inoculation involved a large or small infecting dose (see, for example, Schroeder, 1900), other workers, especially those using naturally infected ticks to set up the primary infection, have found the opposite true (see, for example, Dawson, 1902). Certainly, the latter seems more reasonable in view of the fact that the fatal infection in non-immune animals in nature is accompanied by an enormous infestation of ticks. The two contentions are probably not mutually exclusive, however, because the difference in numbers of parasites in a large and small dose of blood is probably inconsequential compared to the differences in numbers contained in 1 and 500 ticks. A third point which may have a bearing is the possibility of a protective action of the serum injected with the parasites. In line with this, most authors have found it best to use blood from recovered cattle. On the other hand, many authors have stressed the lack of preventive and curative properties of such convalescent serum. The successful demonstration of such properties in canine piroplasmosis, however, suggests that with proper experimental technique, the findings for the cattle parasite may be reversed. Finally, most workers have emphasized the advisability of inoculating only young cows in good health which possess a high general resistance, whereas in nature quite often animals are "run down" in general health and bodily resistance when they contract the disease.

2. *Theileria.* The genus *Theileria,* unlike *Babesia,* is characterized by the fact that the schizonts occur only in the tissues so that peripheral blood is only infective provided it accidentally harbors some endothelial cells containing schizonts (Wenyon, 1926). Furthermore, as was previously indicated, *Theileria,* unlike *Babesia,* is believed to give rise to a sterile immunity, that is, parasites cannot be found in the blood of recovered animals nor can ticks be infected from them. The completeness of recovery has, nevertheless, been

questioned for *T. parva* by Bevan (1924) and van Saceghem (1925 b), and for *T. dispar* by Ed. Sergent *et al.* (1927).

Koch (1903) attempted both immunization and preventive experiments by repeated injections of blood from infected or cured cattle, but Gray (1904) and Theiler and Stockman (1904), in using his methods, obtained very unsatisfactory results. Later, Theiler (1911), after succeeding in transmitting East Coast fever by insertion or injection of infected spleen or lymphatic gland material, noticed that immunity sometimes followed. This led him (1911 b and 1913) to the study of immunization against the disease. As a result of laboratory and field experimentation during the course of which 133,833 cattle had been inoculated in the Transkei, he concluded that immunization was possible, but could only be undertaken with the prospect of conferring immunity on 50-60 per cent, that the immunity was not absolute inasmuch as it failed in 1 per cent of 1,200 experimental infections, and that the best results were procured by inoculating intravenously 2-5 c.c. of spleen and gland pulp from infected cattle, mixed with peptone or aleuronat, and thereafter keeping the treated animals from exposure to the disease for fourteen or fifteen days. Using Theiler's method, Wölfel (1912) found that of a batch of 343 animals, 46 per cent acquired an immunity; but from a further batch of 2,000, although better results were obtained, definite conclusions could not be drawn because the possibility of preëxistent immunes could not be altogether excluded. Spreull (1914) believed that much depended upon whether or not the herd was already infected, because in an extensive series he obtained success in about 70 per cent among clean cattle, 50-60 per cent in slightly infected cattle, and 30 per cent in moderately infected cattle. These investigations indicate that the immunization, although of great value where tick eradication cannot be practised, needs much more work to perfect it. (See recent work of Ed. Sergent *et al.*, 1927).

3. *Anaplasma.* The intracorpuscular bodies, named *Anaplasma* by Theiler, were first described as "marginal points" and considered to be stages in the life cycle of *B. bigemina* by Smith and Kilborne (1893). Later they were considered separate from, but related to, the piroplasms. Their systematic position, however, is decidedly problematical and their place among the parasites is extremely insecure. In fact, although they have been found to be associated with a living transmissible virus, they have not been definitely established as actual micro-organisms. Nevertheless, Theiler (1912) has differentiated two types, *A. marginale* of high virulence and *A. marginale*

var. *centrale* of less virulence. The distinction of virulence has been questioned by Descazeaux and Picollo (1914); and Walker (1915), working in Theiler's laboratory, believed that *A. centrale* could mutate to *A. marginale*.

In spite of the ambiguous nature of anaplasms, it is interesting to note that they are very similar to *B. bigemina* in immunity reactions: an animal having recovered from an acute attack develops a latent or low-grade infection with a concomitant high degree of resistance to superinfection. Theiler (1912), in his immunization studies, found that the injection of the milder *A. m.* var. *centrale* bestowed a partial immunity to the more virulent *A. marginale*. Practically, cattle had to be immunized with both *Babesia* and *Anaplasma,* and extreme precautions had to be exercised to exclude the virulent form of anaplasma and to control severe cases of Texas fever with trypan blue. In the main, Bevan (1913) corroborated these findings, but noted that although trypan blue controlled Texas fever, it apparently made the cattle more susceptible to anaplasmosis, a fact which F. Chambers (1913) verified. Further studies on immunization against anaplasmosis may be found in F. Chambers and Smith (1914), Lignières (1914, 1919 and 1921), Walker (1915), Velu (1922), Ed. Sergent *et al.* (1924 and 1927).

4. MALARIA

The great importance of malaria has led to an enormous bibliography on the general subject of immunity, but hypotheses, speculations and suggestions make up the greater part of it because the rigid host specificity of the human parasite and the inadvisability of experimenting with man make direct controlled experimental studies difficult. The data at hand, however, seem to indicate that resistance to infection among certain individuals exists in malarious districts. There has been, moreover, some work on the avian malarial parasite, and lately, there have been some studies of the human disease on account of its use in the treatment of paresis.

Aside from the early work of Koch (1899) and Ruge (1901) maintaining that the immunity was a result of cure, the work on avian malaria is in agreement in indicating that an acute attack develops into a latent infection of long duration which confers an immunity to superinfection, but that complete disappearance of the parasites allows reinfection. V. Wasielewski (1901 and 1908) showed that birds remained infected for long periods, and Moldovan (1912) showed that canaries, after passing through an acute attack of *Plas-*

modium præcox * were immune to a new infection, whereas upon complete recovery they became reinfected. This work was confirmed by Ed. Sergent and Béguet (1914) working with *Hæmoproteus columbæ,* by Et. Sergent and Hempl (1917) for *P. præcox* where in four out of five birds the immunity to superinfection lasted two and one-half years, by Whitmore (1918) for *P. præcox,* by Mazza (1924) where the immunity lasted four and one-sixth years, by Kikuth and Tropp (1927) and by the author and L. G. Taliaferro (1929).

In attempting to immunize birds, Et. and Ed. Sergent (1910) found that by injecting sporozoites, subjected to 2-23° C. for twelve to forty-eight hours, seven out of twenty-four canaries had only a light or no apparent attack, with a subsequent immunity to superinfection without going through an acute attack. (Sixteen had no infection, one had a heavy infection.) Later, they (1921, 1921 b and c) attempted a long series of immunizing experiments with *P. præcox* in canaries, but the only successful procedures, in addition to the one just mentioned, were the injection of parasites taken from infected birds during the incubation period, which produced tolerance in 21.3 per cent of the cases, and the inoculation of parasites accompanied by doses of quinine (continued daily). Among 965 controls only seven were naturally resistant to infection. The success of these methods was probably due to the injection of such a very small number of parasites (with possibly a lowered virulence) that the latent infection was set up without an apparent acute attack. That a very small dose will suffice has been shown by several investigators. Ed. and Et. Sergent and Catanei (1923) found that whereas it takes the bodies of three infected mosquitoes to produce a severe acute attack, two thirds or three quarters of a single body will produce a slight attack and subsequent immunity. Also, the Sergents (1921 c) reported that one infection produced with blood drawn from another bird during the incubation period showed up three months afterwards. And in the routine infection of hundreds of birds in this laboratory, we occasionally find one which never shows parasites in its blood after injection, although it is infected, since large quantities of its blood when injected into a normal bird will set up the infection.

It has been noted that the acquired immunity to malaria in birds is generally, if not entirely, a question of immunity to superinfection. That such birds (at least in *Plasmodium* infections) are still infected

* See note, page 134. The various bird *Plasmodia* in this section have been listed under the name *P. præcox* where it has been impossible to homologize them with the species described by Hartman (1927 b).

is shown by the fact that their blood is infective to other birds (see page 143 and Whitmore, 1918). Mazza (1923), as mentioned above, found a canary's blood still infective four years and two months after the original infection. Another fact to be noted is that this immunity to superinfection, although of a very high grade (see the work of the author and L. G. Taliaferro, 1929 and 1929 b), is very labile and a large number of conditions which lower the resistance of the body can cause it to flare up into an acute attack (see question of malaria relapse, page 141). As to the basis of the immunity the last-named work (see pages 140 and 232) indicates that it is purely cellular and has no evident humoral basis.

It is very difficult to judge the exact status of acquired immunity to malaria in man because very few controlled experiments have been possible, and it is often difficult to decide whether a given author is considering a given immunity as a resistance to the parasite or to the effects of the parasite. There is considerable evidence, however, that infection, especially with repeated reinoculations by mosquitoes, confers a more or less relative immunity to an acute attack, but there is little or no evidence that any acquired resistance persists for any length of time after complete recovery from infection. (For an extensive bibliography on the subject, see Gill, 1914, and Bass, 1919.) It seems desirable, however, to review in more detail some of the results obtained in the use of malarial therapy in general paralysis because in such cases controlled experiments are possible.

One of the first questions of interest in malarial immunity in man is: Does one attack confer any immunity to a second? The experimental results in the use of malaria for the treatment of paresis do not give a clear-cut answer. Some investigators have found that after a first attack by injection of blood it is often impossible to produce a second attack by injection (Antic, 1925; Nicole and Steel, 1925; Steel and Nicole, 1927; Kirschbaum, 1927). It can, however, occur (Claude, Targowla and Cénac, 1927; Davidson, 1925; and Grant and Silverston, 1926). It may be, just as in avian malaria, that a first attack gives an immunity to superinfection as long as the primary infection lasts, and that second infections are due to the fact that malaria induced by the injection method tends toward more rapid recovery than the naturally acquired disease. It is of particular interest that refractiveness to the malaria induced by injection gives no protection against infection by the bite of infected mosquitoes (Nicole and Steel, 1926; and S. P. James and Shute, 1926). On the other hand, patients who have lived in the tropics since birth are highly refractive to injection malaria and even after successful in-

fection show a high rate of spontaneous recovery (van Loon and Kirschner, 1924), thus indicating that the naturally acquired disease does protect against injection malaria.

Immunity to superinfection may explain at least some of the so-called cases of natural immunity, i. e., where individuals are refractory to several injections without ever experiencing an attack. The originator of the treatment of general paralysis with malaria, Wagner-Jauregg, gave in 1922 an account of 200 transmissions which were carried out by the subcutaneous injection of 1-4 c.c. of blood. In some cases, a first injection was not followed by attacks although a second produced them, and in a few, three or four injections failed to produce attacks. Similar findings were noted by Yorke and Macfie (1924), Grant and Silverston (1924), Dunne (1926), and others. It is, of course, impossible at present to state the reasons for these failures. Technical manipulations may enter as contributing factors. Aside from these, some of the cases of repeated injections seem to be undoubted cases of resistance, of which some may be natural resistance and others may be real latent infections without symptoms or appreciable numbers of parasites in the blood. As to the latter assumption, Lane (1926) suggested, "in some of these cases recalcitrant to injected infection, this [i.e., infection] has actually occurred, has remained latent, and has been made evident by some other factor" and cited, as a possible example, a patient of Yorke and Macfie in whom an injection of *P. malariæ* did not show up by the thirty-ninth day whereupon infection with *P. vivax* was tried with the result that the *P. malariæ* appeared in the blood five and the *P. vivax* thirteen days after the second injection. The postulation of a latent infection to explain the cases of resistance does not answer the question as to why a series of injections will often eventually result in a typical attack or noticeable blood infection. Several possibilities suggest themselves. In some cases, as suggested in the preceding paragraph, there may have been a recovery from a very evanescent initial attack; in others, such matters as dosage of parasites or concomitant adverse factors may overcome the resistance and favor infection or a relapse of the original infection.

5. COCCIDIOSIS

There are many conflicting statements in regard to immunity in animals following infection with coccidia. (For a review of the literature in cats and dogs, see Andrews, 1926). Although some authors maintain that no immunity is acquired as a result of infection,

one experiment of Hall and Wigdor (1918 b) with *Isospora felis* in the dog suggested the contrary. In order to test the point, Andrews (1926) carried out a series of experiments in seven cats and two dogs infected with *I. felis* and *I. revolta* and clearly demonstrated that infection results in an immunity which lasts for at least seven months, and he believes, may last for life. He noted that since the only test for infection is the finding of oöcysts in the stool it was difficult to ascertain whether the immunity was due to complete riddance of the infection or to a very persistent latent or low-grade infection. The latter hypothesis would probably mean that the multiplication cycle continues "at a low ebb" producing few or no oöcysts; this is somewhat supported by the fact that he found an occasional solitary oöcyst in the stools of immune animals, particularly when examined by the concentration method. Inasmuch as examinations were never positive more than ten days after each test inoculation, however, the occasional cyst may only represent one of the inoculated cysts or one from an abortive infection due to the test inoculation. In regard to some of the statements in the literature that immunity to coccidiosis is not acquired as a result of infection, Andrews stated that they may have been based on studies with *Eimeria* which may behave differently from *Isospora*.

V. *Acquired Immunity in Infections with Metazoan Parasites*

Considerable interest has of late been elicited in acquired immunity to metazoan parasites. As its occurrence was doubted for a long time, it seems worth while to list some of the known cases. A critical survey of the literature has been made by Sandground* (1929) from whom the following examples among the helminths are taken.

I. TREMATODES

Among the trematodes Fujinami (1916) and Fujinami and Sueyasu (1917) reported that a horse and some calves became immune to *Schistosoma japonicum* after an infection of short duration. Dogs likewise acquired a high resistance, according to Tanaka (cited by Fujinami). Donham, Simms and Miller (1926) demonstrated a clear-cut case of acquired immunity against the intestinal trematode, *Nanophyetus salmincola*. Infections in the dog produced the so-called "salmon-poisoning"; these, even if light, were generally fatal. A few

*The author wishes to express his gratefulness to Dr. Sandground who kindly allowed him to use his manuscript before it went to press.

cases, however, survived and thereafter possessed a high-grade persistent immunity. The work of Cram (1926) indicated that infections in the natural host, wild carnivores, do not produce severe clinical disturbances.

2. CESTODES

Among the cestodes, there is some evidence that a preëxisting infection in man prevents the development of additional worms and that the immunity to superinfection disappears after expulsion of the parasites (Brumpt, 1927, p. 519, for *Tænia solium* in man; and Joyeux, 1925, for *Hymenolepis* in rats).

3. NEMATODES

Among the nematodes, Ducas (1921) reported a series of experiments on an acquired immunity in rats against reinfection with *Trichinella spiralis* as evidenced by the fact that the larval parasites were arrested in their development and swept out of the intestine before reaching sexual maturity. In view of the results of some other authors, it is highly desirable that these observations be repeated. (See Schwartz, 1917.)

Fernán-Nuñez (1927 and 1927 b) reported the immunization of dogs against infection with *Trichuris vulpis* by injections of the eggs of the parasite at three-day intervals for from seven to twelve months.

One of the most interesting cases of a demonstrated acquired immunity to a metozoan parasite is the recent work of Sandground (1928) on *Strongyloides stercoralis*. Dogs and cats of all ages were found susceptible to the strain which was obtained from a human case. Dogs retained their infection for from two to eleven months, and when they spontaneously lost their infection were refractory to a second infection. As a matter of fact they became refractory to superinfection before they overcame their first infection. Thus, susceptibility to superinfection, as measured by the daily larval output, was lost after a few weeks. Experiments on the mechanism of the acquired immunity, although negative, showed that it was not due to a changed resistance of the skin to penetration because larvæ could not produce infections in immune animals when administered at the back of the tongue or subcutaneously, and that the larvicidal mechanism was not in the blood serum because no evidence could be obtained of *in vitro* activity of blood serum nor of passive transfer of im-

munity. Finally, experiments indicated that when infective larvæ were placed on the skin of an immune dog, the parasites successfully negotiated the skin, migrated through the blood stream and lungs, and probably reached the intestine where they were arrested in their development. Infection with the closely related *S. fülleborni* did not stimulate an immunity against *S. stercoralis* and vice versa.

Recently, Stoll (1928) has reported an acquired immunity in infections of sheep with the common stomach worm *Hæmonchus contortus*. He found that similar to infections with other types of pathogenic organisms, the infection could be of three types: (1) low-grade and perhaps chronic, (2) fulminating and lethal, or (3) self-curative, resulting in a high-grade persistent resistance against further infection with the same species.

4. GLOCHIDIA

In connection with the question of acquired immunity to metazoan parasites, mention should be made of the work of Reuling (1919), who found that after two or three heavy infections of the black bass by the glochidia of *Lampsilus luteola,* the fish acquired an immunity and the glochidia were sloughed off shortly after encysting. The immunity was associated with the acquisition of a lytic property, by the serum of the fish, which acted upon the glochidia *in vitro*.

5. INSECTS

Blacklock and Thompson (1923), working with the myiasis-producing fly, *Cordylobia anthropophaga,* showed that an immunity against the larvæ was developed by man, dogs, monkeys and guinea-pigs. Roubaud and Pérard (1924) attempted to immunize a rabbit against *Hypoderma bovis* by intravenously injecting the glycerinated extract of two larvæ and subsequently testing by implanting larvæ under its skin; they obtained inconclusive results.

Blacklock and Gordon (1927) undertook a most fruitful study of the acquired immunity developed in guinea-pigs against the larvæ of *Cordylobia anthropophaga*. Whether or not the larva was alive at the end of six days after penetration of the skin constituted the criterion of susceptibility or immunity. From a series of experiments with fifty-eight guinea-pigs and 1,650 larvæ, they concluded that following a primary penetration of larvæ into the skin, guinea-pigs acquired an immunity, as Table 30 strikingly shows. The immunity, which is a skin immunity, was localized at first in the areas of skin

TABLE 30

SHOWING THE IMMUNITY ACQUIRED BY GUINEA-PIGS AGAINST LARVÆ OF CORDYLOBIA, AS REPRESENTED BY THE PERCENTAGE OF LARVÆ SURVIVING TO THE SIXTH DAY IN MULTIPLE TESTS. (ON ACCOUNT OF THE LOCAL TYPE OF THE ACQUIRED IMMUNITY, THE LARVÆ WERE ALWAYS PLACED ON THE SAME REGION OF ANY ONE GUINEA-PIG.) (FROM BLACKLOCK AND GORDON, 1927)

Number of test	*Number of regions tested*	*Number of animals used*	*Number of larvæ applied*	*Number of larvæ surviving to the 6th day*	*Percentage of larvæ surviving to the 6th day*
1st	4	58	614	266	43
2nd	4	29	215	22	10
3rd	4	26	210	9	4
4th	4	22	160	2	1
5th	4	18	132	6	5
6th	4	15	117	4	3
7th	4	10	133	0	0
8th	4	5	41	0	0
9th	3	3	18	0	0
10th	2	2	6	0	0
11th	1	1	4	0	0

into which the parasite had previously penetrated or which had been vaccinated with larvæ or emulsions of larvæ; but it tended to spread from the primary focus so that areas which had never been the site of invasion might ultimately become immune; it persisted for at least three months (not tested further); it was found in new skin growing on an abraded immune area; it persisted (in one experiment) in skin transplanted from an immune to a non-immune animal, but not in similar skin kept *in vitro*. The general circulation and the deeper tissues, although necessary for the immunity, took no direct part in the destruction of the larvæ which had penetrated immune areas, since 79 per cent of the larvæ were dead within forty hours, i.e., before they could have penetrated to the deeper tissues.

VI. *Summary*

Some studies on natural immunity indicate that the underlying factors may be largely non-specific, such as thickness of the skin, action of the digestive juices, or physiological conditions associated, for example, with hibernation; they may furthermore be dependent upon phagocytosis or direct parasiticidal properties, unsuitable nutritive materials, and in the case of toxin production, lack of affinity between the body cells and the toxin.

Age immunity, as a natural resistance not associated with past or present infection or contact with derivatives of the parasite, is operative against some of the helminths. In general, it is characteristic of abnormal hosts and is manifested by an increase of resistance as age progresses. Its basis is unknown, but the fact that it is evidenced in abnormal hosts more or less related to the normal hosts suggests that physiological differences of the hosts are less specifically developed in the young and allow development of the parasites, but become more accentuated with age and thereby progressively arrest their development.

Acquired immunity to the protozoan infections has been found to occur in many infections. This may be the result of a low-grade or latent infection, although the infection itself shows tendencies to relapse (*Babesia* and malarial infections); or it may follow supposedly complete recovery from a primary infection (tropical sore, *Theileria* and *Trypanosoma lewisi* infections). It may be extremely race-specific (trypanosome infections in mice cured with drugs).

The mechanism involved in *T. lewisi* infections is associated with the formation in the serum of a lysin and a reproduction-inhibiting activity, with phagocytosis possibly playing a subsidiary rôle. In malarial infections it appears to be due, at least in part, to a heightened phagocytic activity of the reticulo-endothelial system.

Acquired immunity to metazoan parasites may similarly be an immunity to superinfection (*Tænia solium* and *Hymenolepis*) or may persist after the elimination of the parasites (*Strongyloides, Hæmonchus, Lampsilus,* and *Nanophyetus*). A local skin immunity has been found in guinea-pigs against the larvæ of the fly *Cordylobia anthropophaga.*

Little light has been thrown on the mechanism of these immunities, although in *Strongyloides stercoralis* it does not seem due to a changed resistance of the skin or to a larvicidal mechanism in the serum. In glochidial infections (*Lampsilus*) it is associated with a lytic property.

Artificial immunization has been attended with remarkable success among the piroplasms, especially in infections of *Babesia* in cattle, and consists essentially in setting up a low-grade or latent infection, sometimes controlled with trypan blue. Since one attack of oriental sore is likely to confer immunity, sores with their subsequent scars have been avoided on the face by inoculations on less conspicuous parts of the body. Artificial immunization has been more or less unsuccessful in trypanosome infections, although a tremendous amount of work has been done, and in kala-azar.

CHAPTER IX

SEROLOGICAL AND IMMUNOLOGICAL REACTIONS USED IN THE CLASSIFICATION OF PARASITES

I. *Introduction*

One of the striking characteristics of immunological reactions, as has been pointed out in the introductory chapter, is their specificity. This specificity is not absolute but generally shows quantitative differences closely paralleling zoölogical and botanical classifications based on anatomical criteria. Thus, the more closely related two species are, the stronger the "group" reaction between them. For many years investigators have used various immunological reactions to check and extend other biological classifications. It is not within the domain of this work to discuss the basis for the remarkable specificity of various organisms or more particularly of the antigens and test antigens prepared from these organisms. Suffice it to say that such antigenic materials are generally very complex mixtures and their specificity probably resides in the basic structure of their proteins; in the structure of various lipoids and carbohydrates (haptenes and soluble specific substances) which are in themselves not antigenic but which react specifically with specific immune serums and when united with the protein engender specific antibodies; and in the quantitative proportions of the mixtures of various antigens contained in the organism. (See Wells, 1928; Branham, 1928; and Zinsser and Mueller, 1928.)

Zoölogists are prone to disregard all immunological, as well as physiological, methods of classification because a given reaction may be too specific or not specific enough. Thus, when it is found that a single cell strain of trypanosomes through the mediation of immune serums will diversify into a large number of strains which will hold true for a considerable time and will give clear-cut cross-immunity tests among themselves after cure by drugs (see page 120), one naturally becomes skeptical as to the suitability of cross-immunity tests as a means of classifying trypanosomes. All immunological reactions, however, do not exhibit such extreme specificity. Consequently, immunological criteria, just as morphological criteria, should be

studied to see if, on the one hand, they vary too much within what are generally recognized species, or if, on the other hand, they do not differentiate sufficiently among large groups. Keeping this in mind, it is not at all impossible that certain immunological methods may eventually find a place in the identification of species. Furthermore, since immunological specificity depends on chemical structure, a study of immunological relationships would permit the comparison of chemical structure with the taxonomic classification based on anatomical structure. Investigation of such problems is in the formative stage for the parasites. In the following review I have attempted to indicate as far as possible (1) how delicate a given reaction has been found to be, that is, whether it is strain-specific, species-specific, or genus-specific, and (2) what relation there is between the immunological differences and the taxonomic differences based on morphology.

In a sense most of the work on the serology of parasites can be used to illustrate methods of classification. Thus, an investigator in attempting to discover a serological test for a given infection generally considers at once the specificity of the reaction by ascertaining to what extent group reactions exist. The study of the immunological relationships of organisms can best be attained, however, by using antiserums from artificially immunized laboratory animals. By this method, animals, such as the rabbit, in which antibodies are readily produced, can be utilized and immunized until high titer antiserums are obtained. In the present chapter, I shall, therefore, limit the discussion to work directed specifically toward the question of identification and classification of parasites.

II. *Leishmania*

Immunological methods have been used quite extensively to establish the identity or non-identity of various proposed species of *Leishmania* and their relationship to certain insect and plant herpetomonads which bear a great similarity to the cultural forms of *Leishmania.*

The question of the validity of the different proposed species of *Leishmania* infecting man is of considerable interest because they cannot be distinguished morphologically. Originally the following chief types of infection were recognized: (1) kala-azar, a visceral infection produced by *L. donovani,* occurring in India, China, etc.; (2) infantile kala-azar, a visceral infection produced by *L. infantum,* occurring around the Mediterranean; (3) a visceral infection occurring naturally in dogs; (4) oriental sore, a cutaneous infection pro-

duced by *L. tropica;* and (5) American leishmaniosis, a cutaneous infection produced by *L. brasiliensis.* Numbers 1 and 2 are now considered by most authorities as certainly identical and 3 as probably, if not certainly, identical, all being produced by the single species *L. donovani.* Numbers 4 and 5 are considered distinct from *L. donovani,* but opinion is divided in regard to whether they are distinct from each other. For those that consider them distinct, there is some question as to whether *L. brasiliensis* should be a variety of *L. tropica* or a distinct species.

With the lack of morphological criteria, immunological methods, together with other physiological characteristics, have been widely used to establish similarities or differences among these forms. Although single investigators have often obtained clear-cut results, a general survey of the more recent literature indicates that there may be marked differences between the behavior of strains from the same supposed species. Such discrepancies make an evaluation of the data extremely difficult. All of the recent work is consistent, however, in showing that the members of the genus *Leishmania* are a closely related group and are entirely distinct from the genus *Herpetomonas.*

Considering first the work on the relationship of the species of *Leishmania,* the earlier literature dealt with *L. donovani* and *L. tropica.* C. Nicolle and Manceaux (1909) maintained that an initial attack of oriental sore gave a certain degree of immunity against infection with kala-azar in monkeys. Later these authors (1910) stated that, although their experiments were too few to be conclusive, the indications were that oriental sore protected against itself and to a certain extent against kala-azar, but that kala-azar protected against both. These results might be interpreted simply as a group reaction between the two species, or they might be considered to support the suggestion of Manson (cited by Wenyon, 1926) that the two are the same and that oriental sore is a local manifestation of kala-azar in the same sense that vaccinia is a manifestation of smallpox. Later, more definite results were obtained by Laveran (1917) who reported the immunization of a monkey with *L. tropica* and its subsequent injection with three cultures of *L. donovani,* of which it died within a month and a half of the first injection; and, *vice versa,* seven inoculations of two monkeys with cultures of *L. donovani* so that they were evidently infected, and their successful subsequent injection, as evidenced by typical lesions, with *L. tropica.*

Similar tests were employed by Laveran (1914) to establish the identity of *L. donovani* and *L. infantum.* He showed that a monkey (*Macacus cynomolgus*), after recovery from an infection with the

Mediterranean virus (*L. infantum*), was refractory to infection with the Indian virus (*L. donovani*), although the Indian virus produced a lethal infection in another monkey not previously infected.

Attention has already been called to the work of di Cristina (1911 b), who obtained agglutinins (titer 1 : 30) in the serums of rabbits immunized with cultures of *L. donovani.* Since then, this method has been used extensively to study the flagellates. Bandi (1913), after immunizing rabbits with cultures of human kala-azar (*infantum* strain) and canine visceral leishmaniosis, found that the serum of animals immunized with the canine virus agglutinated both strains equally well in dilutions of 1 : 160, but that neither serum was effective against *L. tropica* in dilutions higher than 1 : 70. The belief that both the human and canine visceral infections are produced by the same organism and that the cutaneous parasite is distinct was thereby strengthened.

The identity of the human and canine infections, indicated by Bandi's work, has in the main been verified. Di Cristina and Caronia (1913) studied cross-agglutination and cross-complement fixation in four children who were immunized as follows: a healthy child and a kala-azar patient were immunized with killed cultures of the human virus, and a healthy child and a kala-azar patient with similar cultures of the canine virus. Serums of these children showed both complement fixation and agglutination, but no differences with test antigens prepared from the two sources. A different conclusion was reached by Scordo (1914). Serum from kala-azar patients when mixed with cultural flagellates of both strains, in hanging drops, in small test-tubes and in cultures showed a tendency to be agglutinated with the human strains to a higher degree than with the canine strain. Giugni and Moldovan (see Spagnolio and Giugni, 1914) continued this work and arrived at the same conclusions. Nevertheless, in view of the difficulty in interpreting the phenomena described by Scordo and Giugni and Moldovan, and in view of his own failure to obtain agglutination in cultures with serums of infected or cured animals, Laveran (1917) did not believe their work conclusive.

Pavoni (1914) obtained complete complement fixation with an aqueous extract of an infected spleen both against serum from rabbits immunized with cultural flagellates derived from a visceral infection and against serum from two patients with the cutaneous infection; but this can hardly establish the identity of the visceral and cutaneous forms because the antiserums were obtained from different species of hosts which were immunized in one case and infected in the other. It would have been much clearer to immunize

rabbits with cultural forms of both types and then compare their reactions with test antigens from both. Recent investigators, as will be shown later, have done this and obtained striking differences between the two types of *Leishmania.*

Recently, there has been a revival of interest in the study of phylogenetic relationships of the *Leishmania* by serological methods. Noguchi (1924), after immunizing rabbits by four successive intravenous injections of live cultures at intervals of from five to seven days, obtained clear-cut cross-agglutination tests, but very low titers. Dilutions of 1:10 caused powerful agglutination with the homologous strain, but dilutions of 1:100 were only moderately active. Noguchi was able to separate three varieties of leishmania: (1) *L. donovani* of kala-azar, including the so-called *L. infantum* of the Mediterranean disease which gave identical reactions with it; (2) *L. tropica* of oriental sore with its dermal effects; and (3) *L. brasiliensis* of South American leishmaniosis with its mucodermal predilections. These findings were further checked by the characteristics of the strains when grown on media containing 10 per cent homologous or heterologous immune serum. With homologous immune serum, growth occurred in small clumps, whereas with heterologous or normal serum it was smooth and scum-like. Noguchi's conclusions were in the main checked by Kligler (1925). He used cross-agglutination tests with immune rabbit serum and in addition the leishmanocidal property of immune serum which was manifested by the inhibition of growth of a specific form in tubes containing 20 per cent or more of homologous immune serum. (He refers to this as a lytic property, but until the process is more completely studied, it would seem best to include such phenomena under the more general heading of parasiticidal or leishmanicidal effects.) He studied three strains of *L. tropica* and a strain of the *infantum* type of *L. donovani* and *L. brasiliensis.* Both methods indicated that the different strains of *tropica* are identical but that marked serological differences exist between *L. tropica, L. brasiliensis* and *L. infantum* (*L. donovani*).

Wagener and Koch (1926) have studied the interrelationships of five strains of *Leishmania* and *Herpetomonas ctenocephali* by means of cross-agglutination and the skin test of the senior author. Their agglutination results are given in Table 31, an examination of which indicates that a group relationship existed between all strains of *Leishmania,* but that no affinity was shown between them and *H. ctenocephali.* Furthermore, in practically all cases, the highest agglutination titer was with the homologous strain. These results

TABLE 31

AGGLUTINATION TITERS OCCURRING BETWEEN THE LEISHMANIA AND A HERPETOMONAD AND THEIR HOMOLOGOUS AND HETEROLOGOUS ANTISERUMS (DATA FROM WAGENER AND KOCH, 1926)

Antiserums		*Antigens*					
Number of rabbit	*Species immunized to* *	*L. donovani* (*Tunis strain*)	*L. donovani* (*Peking strain*)	*L. infantum*	*L. tropica*	*L. brasiliensis*	*Herpetomonas ctenocephali*
153	*L. donovani*	40†‡	**100 §**	40	100	80	0
154	(Peking strain)	40			40	80	10
157	*L. infantum*	200	80	300	100	80	10
158		200			200	40	10
151	*L. tropica*	300			500	200	10
152		300	40	40	600	300	10
155	*L. brasiliensis*	200	40	80	500	400	0
156		100			300	300	10
159	*H. ctenocephali*	10	10	10	20	10	80
160		10			20	10	100
Normal (control)		10	10	10	10	10	10

* All immunized rabbits received three intravenous injections.

† The numbers in the table represent the last dilution in which agglutination occurred. (Doubtful readings not included.)

‡ All control tubes (in each set-up) negative.

§ Bold-faced type signifies reaction between homologous antiserum and test antigen.

differ from Noguchi's findings in that the group reactions between the different strains of *L. donovani* and *L. infantum* are not so very much more pronounced than between them and the cutaneous strains. This, the authors suggested, may be due to different techniques, e.g., they used standardized test antigens consisting of killed organisms, whereas Noguchi used living cultures. In any case, the differentiation between the genus *Leishmania* and the genus *Herpetomonas* is very striking. As far as I am aware these authors obtained the highest agglutination titers so far reported, and accordingly their technique is worthy of note. Each rabbit received three intravenous injections (three and five days apart, respectively) of washed living cultural flagellates, and was bled five days later. The test antigen was prepared by washing flagellates from twelve-day N. N. N. cultures, suspending them in 0.9 per cent NaCl in a consistency such that

TABLE 32

SKIN REACTIONS OCCURRING ON SENSITIZED RABBITS AT THE END OF 48 HOURS (FROM WAGENER AND KOCH, 1926)

Number of rabbit	*Species immunized to*	*Reaction on skin of rabbit*	*Antigens used for intradermal injections*							
			L. tropica	*L. brasiliensis*	*L. donovani (Tunis strain)*	*L. donovani (Peking strain)*	*L. infantum*	*Herpetomonas ctenocephali*	*Trypanosoma lewisi*	*Control antigen*
151	*L. tropica*	Necrosis	3 mm.							
		Induration	5 mm.	5 mm.	6 mm.	3 mm.	5 mm.	—	—	—
		Redness	10 mm.	5 mm.	7 mm.	4 mm.	5 mm.	—	—	—
152	*L. tropica*	Necrosis	3 mm.							
		Induration	11 mm.	5 mm.	13 mm.	4 mm.	3 mm.	—	—	—
		Redness	11 mm.	5 mm.	13 mm.	4 mm.	5 mm.	—	—	—
153	*L. donovani* (Peking strain)	Induration	12 mm.	5 mm.	7 mm.	4 mm.	4 mm.	—	—	—
		Redness	14 mm.	5 mm.	7 mm.	5 mm.	5 mm.	—	—	—
155	*L. brasiliensis*	Induration	19 mm.	4 mm.	5 mm.	3 mm.	5 mm.	—	—	—
		Redness	19 mm.	6 mm.	5 mm.	4 mm.	5 mm.	—	—	—
158	*L. infantum*	Induration	12 mm.	4 mm.	8 mm.	4 mm.	6 mm.	—	—	—
		Redness	12 mm.	6 mm.	8 mm.	5 mm.	6 mm.	—	—	—
	Normal control	Induration	—	—	—	—	—	—	—	—
		Redness	5 mm.	—	—	—	—	—	—	—

print was visible through them, adding a drop of toluol and placing in the ice-box. In the tests 0.1 c.c. of test antigen was added to 1 c.c. of the dilution of antiserum, the tubes incubated for two hours at 56° C., read and placed in the ice-box over night to be reread in the morning. The intradermal reaction they used (technique in Wagener, 1923) was a delayed type of reaction which in typical cases appears at the end of twenty-four hours and reaches its height at the end of forty-eight hours. Their results are given in Table 32, which shows no differentiation (group reactions) between the *Leishmania* strains by this reaction, but marked differences between them and *H. ctenocephali* and *Trypanosoma lewisi.*

In a later paper Noguchi (1926) continued his serological study of the *Leishmania* and compared them with various strains of *Herpetomonas* by means of agglutination and complement fixation. Throughout his data, the agglutination part of which is given in Table 33, the clear-cut reactions of different flagellates with homologous immune serums are noticeable. There was no indication of biological relationships between the three species of *Leishmania* and the various strains of *Herpetomonas* from various plants or insects. There were, however, reciprocal reactions between strains of herpetomads from milkweeds and from the insects which feed upon milkweeds. In view of Noguchi's former conclusion that *L. donovani* and *L. infantum* were identical, it is worthy of note that in the present work the two showed marked differences, although there was still a group affinity between them (cf. results of Wagener and Koch). Almost identical findings were obtained by Noguchi using complement fixation.

Recently the Rieckenberg phenomenon has been applied to the study of *Leishmania,* Burowa (1928), using the serums from mice inoculated with *L. donovani* and *L. tropica,* differentiated the two species. The validity of the test was weakened because of the fact that normal mouse serum also gave the reaction, although to a lessened degree. Chodukine and Soffieff (1928), using the serums from mice, guinea-pigs and rabbits immunized with different strains, were unable to differentiate *L. tropica, L. donovani* and two canine strains. Lytic and agglutination tests, however, differentiated between *L. tropica* and *L. donovani* and indicated that the canine strains were identical with the latter.

An interesting use of serological tests was made by Adler and Theodor (1926) who attempted to identify a given protozoön, originally isolated from a vertebrate host, with possible stages of this protozoön in invertebrate hosts captured in nature. Thus, agglutina-

TABLE 33

AGGLUTINATION TESTS WITH CULTURAL LEISHMANIA, TRYPANOSOMA AND HERPETOMONAS (REARRANGED FROM NOGUCHI, 1926)

Source of flagellate	*Antiserums (1:21)*										
	Anti-tropica	*Anti-brasiliensis*	*Anti-infantum*	*Anti-donovani*	*Anti-oncopelti (Strain from O. fasciatus)*	*Anti-lygæorum (Strain from L. kalmii No. 2)*	*Anti-culicidarum (Strain from A. quadrimaculatus)*	*Anti-muscidarum (Strain from M. domestica)*	*Anti-media (Strain from Calliphora? No. 1)*	*Anti-parva (Strain from Calliphora? No. 2)*	*Normal rabbit serum*
Oriental sore	++++	+	+	—	—	—	—	—	—	—	—
Espundia	—	++++	—	—	—	—	—	—	—	—	—
Kala-azar *infantum*	+	—	++++	+	—	—	—	—	—	—	—
Kala-azar *donovani*	—	—	++	++++	—	—	—	—	—	—	—
Oncopeltus fasciatus	—	—	—	—	++++	+	—	—	—	—	—
Oncopeltus sp.?	—	—	—	—	++++	+	—	—	—	—	—
Lygæus kalmii No. 1	—	—	—	—	++++	+	—	—	—	—	—
Lygæus kalmii No. 2	—	—	—	—	+	++++	—	—	—	—	—
Anopheles quadrimaculatus	—	—	—	—	—	—	++++	—	—	—	—
Culex pipiens	—	—	—	—	—	—	++++	—	—	—	—
Musca domestica	—	—	—	—	—	—	—	++++	—	—	—
Calliphora sp.? No. 1	—	—	—	—	—	—	—	—	++++	—	—
Calliphora sp.? No. 2	—	—	—	—	—	—	—	—	—	++++	—
Asclepias syriaca No. 46	—	—	—	—	+	++++	—	—	—	—	—
Asclepias syriaca No. 213	—	—	—	—	++++	++	—	—	—	—	—
Asclepias nivea	—	—	—	—	++++	+	—	—	—	—	—
Ctenocephalus canis (*H. ctenocephali*)	—	—	—	—	—	—	—	—	—	—	—
Rana pipiens (*Trypanosoma rotatorium*)	—	—	—	—	—	—	—	—	—	—	—

++++ rapid, complete precipitation, agglutination involving both cytoplasm and flagella.
+ definite effect on flagella, which are twisted and adhere to one another.

tion tests indicated that *L. tropica* and *Herpetomonas papatasii* are identical.

III. *Trypanosomes*

Although the genus *Trypanosoma* can be divided into large groups by morphological characteristics, the individual species themselves are frequently quite indistinguishable by such means. Various biological criteria have therefore been adopted, such as host species, type of development in the invertebrate and vertebrate hosts, susceptibility of various hosts, etc.

Supplementary to these, certain investigators, particularly the French, have used immunological methods. Of these, the most widely used has been the cross-immunity test, which was advocated by Laveran and Mesnil in 1905. This entails the use of goats, sheep or to a less extent cattle, all of which offer a high resistance to trypanosomes and acquire an immunity following recovery. The salient features of the technique, as described by Laveran and Mesnil (1912), are as follows: A sheep or goat is infected with a given strain of trypanosomes; when recovered it is reinoculated with the same strain of parasites; and when again recovered, it is inoculated with the strain to be identified. Subsequently, if it becomes infected, the two strains are considered distinct, and if not, they are considered identical. To be experimentally perfect every step in the procedure, including the initial infection, has to be demonstrated by animal subinoculations since the parasites are too few to be demonstrated microscopically; in the case of recoveries amounts as large as 30 to 50 c.c. need to be used to guarantee non-infectivity. If the two strains are distinct, a reciprocal test must then be carried out because failure to infect may have been due to a low virulence in the second or test strain. The second strain is accordingly used as the initial infecting strain and the first strain used as the "runner up."

By this method, Laveran and Mesnil (1905) and Laveran (1911 c and 1912 b) differentiated *T. evansi* from *T. brucei* and *T. hippicum*, *T. congolense* from *T. dimorphon* and *T. pecorum*, and *T. rhodesiense* from *T. gambiense*. In addition they established the identity or non-identity of the trypanosomes from surra (*T. evansi*) or surra-like diseases occurring in various localities. Ed. Sergent, Lhéritier and Belleval (1915) believed that the test was reliable enough to justify their proposing a new species, *T. marocanum*, as distinct from *T. berberum*, *T. equiperdum* and *T. soudanense*.*

* There seems no point in reviewing all of the papers on the classification of various trypanosome strains by this method. Some of the more important

In evaluating the method it appears, if anything, too specific. Thus, many protozoölogists do not recognize all of the foregoing species, and Wenyon (1926) believes them to be basically five species —viz., *T. gambiense, T. brucei, T. congolense, T. evansi* and *T. equiperdum,* of which *T. rhodesiense* is considered a human strain of *T. brucei, T. pecorum* and *T. dimorphon* (at least in part) as *T. congolense,* and *T. soudanense, T. marocanum, T. berberum* and *T. hippicum* as races of *T. evansi.* Furthermore, the method has been criticized in view of the peculiarly specific cross-immunity tests in mice after drug treatment, although it has not been established that the degree of specificity after cure in goats, sheep and cattle is directly comparable to that in mice after treatment with drugs.

In any case, some of the work on mice is very interesting. The upshot of a prodigious amount of work seems to show that the cross-immunity test in mice following treatment with drugs is so specific as to differentiate passage from relapse strains whether these be produced by drug treatment or by antibodies—even when originated from a single cell strain (see page 120). The whole question has been recently reopened by Kroó (1925 and 1926), who concluded that the test was strain-specific, and hence, too delicate for the differentiation of species. In his first paper, three strains of *T. brucei* ("Prowazek," "Ferox" and "Pasteur Institute") all gave cross-immunity tests in mice after treatment with potassium tartrate. A typical experiment is well illustrated in Table 34. Twenty-four mice were infected with the "Prowazek" strain; on the third day of the infection they were treated with 0.2 c.c. per 20 grams mouse of potassium antimony tartrate (1 : 1,000); the day following they were injected with the three strains (eight mice to each strain) with the result that the eight receiving the heterologous Pasteur Institute strain (mice 17-24) became infected and died on the eighth day, the eight receiving the heterologous Ferox strain (mice 9-16) similarly became infected and died on the seventh and eighth days, whereas of the eight receiving the homologous Prowazek strain (mice 1-8) only two showed any infection and none were dead by the eighth day. In the second paper, the Prowazek strain was subinoculated from mice into an ox, two sheep, a dog, rabbit and guinea-pig; four weeks later trypanosomes from each animal, when reinoculated into mice, showed themselves to be all different by cross-immunity tests. Such results are essentially

are: Laveran and Mesnil (1905), Laveran (1909, 1911, 1911 b and c, 1912 b and c), Mesnil and Ringenbach (1911, 1914), Mesnil and Leger (1912), Roudsky (1912, dealing with *T. lewisi* and *T. duttoni* in rats and mice), Bruce, Hamerton, Watson and Bruce (1914), Ed. Sergent and Donatien (1924).

TABLE 34

CROSS-IMMUNITY TESTS WITH DIFFERENT STRAINS OF T. BRUCEI IN MICE AFTER CURE WITH DRUGS (FROM KROÓ, 1925)

Mouse	Day							
	1	2	3	4	5	6	7	8
1	Infected with strain "Prowazek"	+	++	0 Infected with strain "Prowazek"	0	0	0	0
2		+	++	0	0	0	0	+
3		+	++	0	0	0	0	0
4		+	++	0	0	0	0	0
5		+	++	0	0	0	0	0
6		+	++	0	0	0	0	0
7		+	++	0	0	0	0	0
8		+	++	0	0	0	0	+
9		+	++ Potassium antimony tartrate 1 : 1,000, 0.2 c.c. per 20 gm. mouse	0 Infected with strain "Ferox"	+	++	D	
10		+	++	0	+	++	D	
11		+	++	0	+	++	D	
12		+	++	0	+	++	++	D
13		+	++	0	+	++	D	
14		+	++	0	+	++	++	D
15		+	++	0	+	++	D	
16		+	++	0	+	++	D	
17		+	++	0 Infected with strain "Pasteur Instit."	+	++	D	
18		+	++	0	+	++	D	
19		+	++	0	+	++	D	
20		+	++	0	+	++	D	
21		+	++	0	+	++	D	
22		+	++	0	+	++	D	
23		+	++	0	+	++	D	
24		+	++	0	+	++	D	

similar to the earlier work where investigators found differences between strains known to have the same origin. The dissociation of the strains takes place in animals known to produce antibodies, and the resulting differences probably represent antibody-resistant strains. Inasmuch as growing the trypanosomes in various laboratory animals may induce biological characteristics of greater significance than the natural differences between species, the method may be adequate where it is possible to use it on the species in nature. Thus, as previously pointed out, Braun and Teichmann (1912 c) found that immunizing with one species of trypanosome would show a group immunity with several other species, and yet immunizing with an antibody-resistant variant of the one species would not protect against the original strain of the same species.

Besides the cross-immunity method, other *in vivo* tests have been used, but to a less extent. Laveran and Mesnil (1906) called attention to the use of the protective action of a given serum when injected together with its specific trypanosome into mice. The test was ren-

dered more adaptable when Laveran (1911) found that animals retained this property over long periods (seventeen months in the case of a goat cured of its infection, two years and six months in the case of a sheep), and when Laveran and Mesnil established that serum from subacute or chronic infections possessed a like property.

Recently, Kokawa (1927) has attempted to use anaphylaxis in guinea-pigs as a method of differentiating species and has found that *T. lewisi* could be thus differentiated from the pathogenic forms, but within the latter group no differentiation could be obtained between *T. gambiense* and *T. equiperdum.*

In addition to the *in vivo* tests, various serological tests have been used. Some (e.g., the blood-platelet reaction), like cross immunity in mice after treatment with drugs, are only strain-specific, and hence too delicate; but on the whole, most of them were discredited from the start as being genus-specific and not species-specific. With the recent tendency of authorities like Wenyon (1926) to combine many of the formerly recognized species, however, it is possible that they may be of great value if reëxamined, particularly if used in conjunction with modern absorption methods. For example, Robinson (1926), using the complement fixation reaction, found that the serums of animals infected with *T. congolense* reacted with the homologous test antigen, but not with *T. equiperdum,* whereas a group reaction occurred between *T. brucei* and *T. equiperdum.*

The phenomenon of "attachment" was first applied to the differentiation of trypanosome species by Levaditi and Mutermilch (1910 b) and is described on page 132. With it a strain of *T. brucei, T. togolense* (another strain of *T. brucei*), *T. dimorphon* (partly *T. congolense*) and *T. gambiense* were differentiated. The next year Laveran and Thiroux (1911) decided that the method was not so sensitive as cross immunity, and Levaditi and Mutermilch (1911) concluded that the reaction showed group reactions similar to the findings of Leger and Ringenbach (1911 and 1912) for trypanolysins. In view of the recent tendency to recognize fewer species, as pointed out above, the reaction may be found of value for species differentiation.

The interest in the use of *in vitro* trypanolysis as a method of classifying and identifying trypanosomes dates from the cross-lytic tests of Levaditi and Mutermilch (1909). Later Leger and Ringenbach (1912) found that the trypanolytic serums exhibited well-marked group reactions, but generally could be relied upon to differentiate species recognized as being related, such as *T. evansi* and *T. equinum* (Table 35). Although this method promised many interesting results

TABLE 35

CROSS TRYPANOLYSIS EXPERIMENTS (REARRANGED FROM LEGER AND RINGENBACH, 1912)

Nature of infection in the guinea-pigs furnishing immune serum			*Virus used in in vitro tests* *									
Species	*Number of days after inoculation*	*Stage of infection*	*T. brucei* #1	*T. brucei* #2	*T. togolense (nagana of Togoland)*	*T. brucei (?)*	*T. equinum*	*T. gambiense*	*T. congolense*	*T. evansi*	*T. rhodesiense*	*T. dimorphon*
T. brucei #1	12	First acute rise		++	++	++		—	—		—	
T. equinum	4	Beginning of first rise	—		+		+			—		+
	9	First crisis	—				+++	+		+	+	
	14	Beginning of second rise	—				++	—	—	+++	—	—
T. gambiense	6	First rise			—		—	+		—	+	—
	10	First crisis	—				—	++		—	+	
	68	End of second rise			—			+	—		+	
T. congolense	13	Beginning of first rise					—	—	+	—	—	—
	25	End of first rise						—	++			+
T. pecaudi	13	First rise		—			—	—	—	—	—	—

+++ = Rapid destruction (1-2 hours); ++ = Slow destruction (2-4 hours); + = Deformation (4 hours); — = No action.

* Of the species listed, Wenyon (1926) considers *T. togolense* as *T. brucei; T. rhodesiense,* a human strain of the same; *T. dimorphon* in part as *T. congolense;* and *T. equinum* as possibly a strain of *T. evansi.*

TABLE 36

AGGLUTINATION TITERS OBTAINED WITH SERUMS FROM INFECTED RABBITS AND MICE AND FROM VARIOUS NORMAL ANIMALS AGAINST T. CONGOLENSE, T. BRUCEI, T. EQUIPERDUM (2 STRAINS) AND T. GAMBIENSE
(REARRANGED FROM DATA OF MATTES, 1912)

Animal	*Infected with*	*Day of infection*	*Test material*	*T. congolense*	*T. brucei*	*T. equiperdum (Algiers)*	*T. equiperdum (Europe)*	*T. gambiense*
Rabbit	*T. congolense*	15	Serum	**500**	300	200	200	
		40	Serum	**5000**	6000	5000	5000	
		59	Serum	**9000**	9000	8000	8000	8000
Mouse	*T. congolense*	40	Serum	**2000**	2000	1500	1500	
		75	Serum	**7000**	6000	7000	1000	6000
Rabbit	*T. brucei*	12	Serum	800	**1500**	1000	1000	800
		19	Serum	1500	**3000**	2000	2000	1500
		19	Peritoneal exudate	1500	**3000**	2000	2500	1500
		19	Pleural exudate	1500	**3000**	2000	2000	1500
		19	Heart cavity exudate	1500	**3000**	2000	2000	1500
Rabbit	*T. equiperdum* (Algiers)	14	Serum	1000	2000	**3000**	2000	2000
		20	Serum	5000	5000	**6000**	6000	5000
		35	Serum	9000	9000	**12000**	10000	9000
Rabbit	*T. equiperdum* (Europe)	12	Serum	300	500	300	**2000**	300
		19	Serum	1000	1000	1000	**2500**	1000
		32	Serum	3000	4000	4000	**5000**	3000
Mouse	*T. gambiense*	15	Serum	200	300	400	400	**800**
		31	Serum	1000	1000	1500	1500	**2000**
Human	Normal		Serum	5	5	5	5	5
Horse	Normal		Serum	50	100	80	100	80
Cattle	Normal		Serum	50	80	50	100	30
Sheep	Normal		Serum	20	20	10	10	10
Goat	Normal		Serum	10	20	5	10	10
Pig	Normal		Serum	10	10	10	5	5
Rabbit	Normal		Serum	10	5	10	10	5
Guinea-pig	Normal		Serum	10	10	5	5	5
Mouse	Normal		Serum	10	5	5	5	5

Bold-faced type = Reaction between homologous antiserum and test antigen.

it has not been developed to any great extent. Incidentally, Leger and Ringenbach reported lysis of both *T. gambiense* and *rhodesiense* by the same serum, although Mesnil and Ringenbach (1911) had previously obtained differences in lytic effects. The finding of a group reaction in the trypanolysins is interesting in view of the fact discussed on page 118 that a given trypanolytic serum is not active against a relapse strain of organisms which represents a biological variant of the same species which stimulated the production of the lysin. In other words, just as in the case of cross-immunization, discussed previously, some evidence indicates that the lysins are strictly strain-specific and other evidence indicates that they are not strictly species-specific. In comparing the *in vivo* action of immune serum, the phenomenon of attachment and that of trypanolysis on *T. gambiense* and *T. rhodesiense,* Mesnil and Ringenbach (1911) concluded that trypanolysis gave the most constant results; but in testing the latter two methods Laveran and Nattan-Larrier (1912) found neither very efficient.

Although no extensive use has been made of the method of agglutination various workers have found differences in the titer between homologous, as compared to heterologous, antiserums and test antigens. (Cf. the extensive work of Mattes, 1912, shown in Table 36.)

In summing up the available evidence various immunological reactions may prove valuable aids in studying the identification, classification and various relationships of the trypanosomes, but these need to be reworked both in light of their fundamental nature and in the application of more modern methods of technique. Furthermore, the fact that the most specific reactions, such as cross immunity in mice after drug treatment, may differentiate antibody variants and at the same time show group reactions between species, indicates that such reactions may be of value in the study of original species in nature, but may be valueless for the variously modified strains kept in the laboratory.

IV. *Piroplasms*

A certain amount of work has been done with cross-immunity tests for the identification or differentiation of so-called species of piroplasms, and in spite of the method's lack of finality, the work has at least served to direct the attention of systematists to specific problems of classification. The following examples are representative of the results obtained. Theiler (1904 b) used it as one of the criteria to

differentiate the genus *Theileria* (East Coast fever of cattle) from that of *Babesia bigemina* (Texas fever of cattle), and later (1912), to differentiate anaplasmosis from Texas fever. Among the recognized species, Stockman and Wragg (1914) found that cattle recovering from *B. bigemina* were susceptible to *B. bovis* (*P. divergens*); Nuttall and Strickland (1912) and du Toit (1919) similarly found a difference in susceptibility to *B. equi* and to *B. caballi;* and Brumpt (1920) differentiated *B. argentina* from *B. bovis,* although many authors consider the two as probably the same. Finally among the more closely related strains, Laveran and Nattan-Larrier (1913) differentiated between the French and North African strains of *B. canis.* This strain specificity (as pointed out by Laveran) may account for the results of Ciuca (1913) who found that dogs having recovered from the Tonkin virus (*B. canis*) were susceptible to Nuttall's South African virus. Likewise, Rosenbusch and Gonzalez (1925) found strain differences in the babesias (causing tristeza) from various localities of Brazil.

V. *Malaria*

The author has been unable to find any extensive use of immunological methods in the classification of the malaria parasites. Recently, however, Hartman (1927 b), in describing three species of malaria (*Plasmodium præcox, P. cathemerium* and *P. inconstans*) from the English sparrow, noted that infection with one prevents reinfection with itself, but not infection with the other two, a fact which he used to confirm the morphological evidence that the three were distinct species.

VI. *Helminths*

1. GENERAL STUDIES ON CLASSIFICATION

A large number of the investigations reviewed in Chapter II bear on the general subject of the biological relationships of various worms as evidenced by group reactions in serological tests, but since they are noted because of possible sources of error in diagnosis and generally deal with very obvious relationships, they need not concern us here.

One of the first papers dealing with the specific question of biological relationships as evidenced by cross serological tests is that of Kolmer, Trist and Heist (1916) on complement fixation in intes-

TABLE 37

CROSS COMPLEMENT FIXATION TESTS WITH RABBIT (IMMUNIZED WITH SALINE EXTRACTS OF THE PARASITES) ANTISERUMS AND TEST ANTIGENS OF INTESTINAL PARASITES OF THE DOG (DATA FROM KOLMER, TRIST AND HEIST, 1916)

Antiserums	*Test antigens**				
	T. serrata	*D. caninum*	*T. canis*	*T. vulpis*	*D. renale*
Tænia serrata	<.001 †	.005	.1	.1	.05
	<.001	.01	—	.2	.1
Dipylidium caninum	.005	.005	.05	.1	.05
Toxocara canis	.1	—	.005	—	.05
	—		.01		.01
Trichuris vulpis	.1	—	.05	.005	.05
	.1	—	.05	.005	.1
Dioctophyme renale	.2	.2	.01	—	.001
	—	—	.01	—	<.001

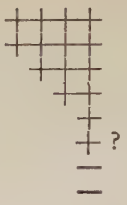
* Test antigens were saline extracts of the parasites.

† The titer is expressed as the smallest fraction of a c.c. giving a positive reaction (generally about 25 per cent inhibition of hemolysis). The upper figure represents the titer with active serum and the lower figure with serum inactivated at 62° C. for 30 minutes.

Bold-faced type = Reaction between homologous antiserum and test antigen.

tinal helminth infections of dogs. Rabbits were immunized by intravenous injections of saline extracts of *Tænia serrata, Dipylidium caninum, Toxocara canis, Trichuris vulpis* and *Dioctophyme renale;* cross complement fixation tests were run with the same type of saline extracts as test antigens. The results are given in Table 37, an examination of which indicates that, with the exception of the *Dipylidium* antiserum which reacted as well with either tapeworm, each antiserum reacted most strongly with its homologous test antigen; that an unquestionable and marked relationship existed between the two tapeworms and that there was only a more distant relationship between *T. canis* and *D. renale*. The remaining cross reactions, the majority of which showed fixation with larger amounts of serum (0.05 to 0.2 c.c.), will not be discussed because the rabbits were not tested for non-specific complement fixation before immunization and might readily have shown non-specific fixation with that quantity of serum.

The relationship of various ascarids, as indicated by the precipitin test, was studied by Schwartz (1920). Antiserum from rabbits immunized with saline extracts of *Ascaris lumbricoides* from pigs, when tested against homologous and heterologous test antigens, gave the following results:



The relationship of the same forms was studied by anaphylaxis in guinea-pigs. Although the results were variable, the tests with guinea-pigs sensitized with *A. lumbricoides* and later given intoxicating doses of the various species pointed to much the same conclusions as did the precipitin tests. The serological identity of *A. lumbricoides* from man and the pig was further corroborated by Bakker (1921) in a complement fixation study.

TABLE 38

RESULTS OF CROSS PRECIPITIN ("RING TEST") TESTS WITH RABBIT ANTISERUMS AND VARIOUS TEST ANTIGENS (FROM HEKTOEN, 1926)

Source of test antigen	*Antiserums*					
	Ascaris lumbricoides			*S. dentatus*	*G. scutalium*	*M. hirudinaceus*
	Extract	*Albumen*	*Globulin*			
Ascaris lumbricoides (Extract)	**10000**	400	0	0	0	0
(Albumen)	6000	**6000**	0	0	0	0
(Globulin)	4000	0	**10000**	0	0	0
Stephanurus dentatus (hog)	0	0	0	**1400**	0	0
Gongylonema scutatum (beef)	0	0	0	0	**1000**	0
Macracanthorhynchus hirudinaceus	0	0	0	0	0	**400**
Ascaris columnaris (skunk)	3000	200	0	0	0	0
Parascaris equorum	500	300	0	0	0	0
Strongylus edentatus (horse)	0	0	0	500	0	0
Toxocara canis (dog)	0	200	0	0	0	0
Setaria equina (horse)	0	200	0	0	0	0
Cysticercus bovis	0	0	0	0	0	0
Tænia saginata	0	0	0	0	0	0
Oxyuris equi	0	0	0	0	0	0
Dictyocaulus filaria (sheep)	0	0	0	0	0	0
Passalurus ambiguus (rabbit)	0	0	0	0	0	0
Œsophagostomum columbianum (sheep)	0	0	0	0	0	0

Bold-faced type = Reaction between homologous antiserum and test antigen.

It would be interesting to continue the work of Schwartz and to make an extensive study of the relation of anaphylaxis to zoölogical classification. The possibility of peculiar group reactions is indicated by the fact that Parisot and Simonin (1920) and Simonin (1920) reported the production of anaphylactic shock with an ordinarily non-fatal dose of *Ascaris* perienteric fluid in guinea-pigs previously sensitized with hydatid fluid.

Hektoen (1926) also used the precipitin method for the study of the relationship of various animal parasites. Rabbits were immunized with saline extracts of powdered parasites and their antiserums tested against the same type of test antigen by means of the ring test. His results are given in Table 38, from the data of which he concluded that the precipitin reactions in general follow the law of species specificity and that the only exception is the case of *Setaria equina* which in its classification is far removed from the ascarids.

2. TISSUE SPECIFICITY

Working in this laboratory, Canning (1929) has carried the study of cross serological tests much farther in that he has investigated the question of tissue specificity. Rabbits were immunized with saline suspensions of dried powdered isolated tissues of *Ascaris lumbricoides* from the pig. They were then tested for precipitins (ring test) against their homologous and heterologous isolated tissue and whole worm test antigens not only of *Ascaris* but also of other species of ascarids. Without exception, when the *Ascaris* tissue antiserums were tested against various *Ascaris* tissue test antigens the highest titer was obtained when a given antiserum was tested against its homologous test antigen. The greatest specificity was shown by the cuticle, and in a decreasing degree by the egg, muscle, sperm and intestine in the order mentioned, as is clearly shown in Table 39. Furthermore, when the same antiserums were tested against tissue test antigens from other species, the following seemed to be indicated: the egg test antigens showed the greatest species specificity; sperm test antigens were least species-specific; and cuticle test antigens were peculiar in that there was absolute specificity between the mammalian and avian ascarids, but no specificity between the different mammalian ascarids, as is shown in Table 40. The finding of such clear-cut tissue or organ specificity in *Ascaris* is in itself of tremendous interest in view of the fact that in mammals organ specificity is rare. Canning feels that this difference is probably due to the fact that the organs or tissues used in his work had a single germ-layer ancestry, which is not the case in

TABLE 39

AVERAGE TITERS * OBTAINED WITH ANTI-TISSUE SERUMS (FROM RABBITS IMMUNIZED WITH VARIOUS TISSUES FROM ASCARIS LUMBRICOIDES) AGAINST TISSUE TEST ANTIGENS FROM ASCARIS LUMBRICOIDES (FROM CANNING, 1929)

Antiserum	*Test antigens*				
	Egg	*Sperm*	*Muscle*	*Intestine*	*Cuticle* †
A Anti-egg	**4000**	500	1100	100	100
B Anti-sperm	1300	**2400**	900	300	300
C Anti-muscle	100	70	**2900**	700	100
D Anti-intestine	100	100	1200	**1200**	9
E Anti-cuticle	50	80	100	50	**3500**

* The numbers in this table represent the average titer of the highest reacting dilution of the test antigens. Only differences of a hundred are noted except when the average is less than 100. Between 6 and 14 tests were done in each case.

† The heterologous tests with cuticle should be even lower, because with later improvement in technique in separating the cuticle from the rest of the body, no reaction was obtained other than with the homologous antiserum.

Bold-faced type = Reaction between homologous antiserum and test antigen.

TABLE 40

AVERAGE TITERS * OBTAINED WITH ANTI-TISSUE SERUMS (FROM RABBITS IMMUNIZED WITH VARIOUS TISSUES FROM ASCARIS LUMBRICOIDES) AGAINST TEST ANTIGENS FROM VARIOUS HELMINTHS (FROM CANNING, 1929)

Anti-tissue serum (*A. lumbricoides*)	*Tissue test antigen* (*various species*)	*Species from which test antigens were derived*					
		A. lumbricoides	*Parascaris equorum*	*Toxocara canis*	*Ascaridia lineata*	*Ascaridia columbæ*	*Macracanthorhynchus hirudinaceus*
A Anti-egg	Egg	**2900**	400	400	36		0
B Anti-egg	Whole worm	3400	2400	1600	**200**	300	40
C Anti-sperm	Sperm	**1900**	500	300	300		200
D Anti-sperm	Whole worm	3400	3200	1100	1000	800	60
E Anti-sperm	Egg	2500	1100	1100	2500		66
F Anti-muscle	Muscle	**3400**	1000	900	100		
G Anti-intestine	Intestine	**1100**	200				
H Anti-cuticle	Cuticle	**3600**	3600	3600	0		

* The numbers in this table represent the average titer of the highest reacting dilution of the test antigens. Only differences of a hundred are noted except when the average is less than 100. Between 3 and 12 (generally at least 9) tests were done in each case.

Bold-faced type = Reaction between homologous antiserum and test antigen.

mammals, and to the further fact that the more nuclear material there is compared to the cytoplasm, the less the specificity. In support of this later contention the sperm antiserums showed wide group affinities, while the egg antiserums were more specific—although both were of the same embryonic parentage. In fact, this might have been predicted in the light of current conceptions of embryonic development which maintain that the nuclei contain the general characteristics of the species and are more or less similar throughout the organism, whereas the segregation of specific cytoplasmic materials at each cleavage is connected with the differentiation of the blastomeres and subsequent layers. Finally, he believes that the cuticle shows practically an absolute specificity to the other tissues of *Ascaris lumbricoides* and yet is immunologically identical for the various mammalian ascarid cuticles studied because it is a non-nucleated secretion product of the ectoderm. The outstanding feature of this very interesting work is that test antigens prepared from different parts of *Ascaris* vary in regard to their specificity, a fact which suggests that future classifications may be made by using one tissue to show group reactions and another to show specific differentiations.

VII. *Summary*

Serological and immunological reactions, although highly specific, generally show quantitative differences closely paralleling zoölogical and botanical classifications based on anatomical criteria. The specificity and quantitative differences of these reactions probably reside in the following factors of the antigenic mixtures derived from the different organisms: the basic structure of their proteins; the structure of various lipoids and carbohydrates which in themselves are not antigenic, but which react specifically *in vitro* and which become antigenic in combination with proteins; and the quantitative proportions of the various antigenic substances.

In general, zoölogists do not accept physiological characteristics, including immunological and serological reactions, as valid criteria for classification. Past experience indicates that many of these reactions are too specific and others are too non-specific. Thus, cross immunity after drug or serum therapy in the trypanosomes seems too specific for a basis for classification, since it has been shown that a single cell strain of trypanosomes may split up, under the influence of drugs or antiserums, into a large number of strains that give sharp cross-immunity tests. Similar strain specificity seems to be characteristic of the trypanosomes and piroplasms in cross-immunity tests

following natural recovery. A puzzling feature is evidenced, at least in trypanosome work, by the fact that various immunological differences seem to be greater between antibody or drug variants of a given strain of organism than between recognizedly different but related species. Agglutination and complement fixation tests, which have recently been used with the *Leishmania* and related flagellates, are undoubtedly genus-specific and species-specific, but they too may be strain-specific.

Regardless of the feasibility of using such reactions as a basis of classification, the fact that immunological specificity does rest on chemical structure makes it very interesting to compare immunological specificity with zoölogical classification. Various serological studies on the helminths indicate that the two are closely parallel, with occasional marked exceptions. A further refinement of such studies makes use of the isolated tissues of *Ascaris* where there appears to be a high degree of tissue specificity and where different tissues exhibit markedly different group reactions when tested against tissue or whole worm extracts of related helminths.

APPENDIX

CATALOGUE OF THE PARASITES CONSIDERED IN THE PRESENT VOLUME

In recent years the intensive interest in the classification and nomenclature of parasites has often resulted in frequent changes of the names of well-known forms. This has been due partly to questions of priority in settling the valid name and partly to changes in the concept of classification (as when a large genus is split into several smaller genera). In any event, it is becoming increasingly difficult for investigators, not primarily parasitologists, to recognize the names of even the commoner forms. In reviewing the work in the present volume I have endeavored to use the names which are generally accepted by parasitologists at the present time. Most of the forms studied with immunological techniques are well known and have been easy to identify, but a few, it is very possible, have not been correctly identified. To aid those readers who consult the original articles, I have appended lists of the parasites mentioned in the preceding chapters, showing (a) their presumably valid names, which are used in this book, (b) their common synonyms and (c) their common hosts. This list is not intended to be complete, but simply to contain the items of probable value to the present readers. In preparing it, I have made extensive use of Wenyon (1926), Yorke and Maplestone * and the series of Key-Catalogues of parasites by Stiles and Hassall and have received much valued advice from Dr. Albert Hassall and other members of the United States Bureau of Animal Industry.

I. Parasitic Amœbæ

Name used in present volume	*Common synonyms*	*Common hosts*
Endamœba coli	*Endamœba* or *Entamœba hominis*	Man
E. histolytica	*Endamœba* or *Entamœba dysenteriæ, minuta* or *tetragena*	Man
Endolimax nana	*Endolimax intestinalis, E. phagocytoides; Entamœba* or *Endamœba nana*	Man
Iodamœba williamsi	*Iodamœba* or *Entamœba bütschlii;* "Iodine cysts"	Man

* *The Nematode Parasites of Vertebrates,* 1926. Philadelphia. Pp. 536.

II. Flagellates

Name used in present volume	*Common synonyms*	*Common hosts*
Chilomastix mesnili	"Cercomonas"	Man
Cryptobia helicis	*Trypanoplasma helicis*	Snail
Giardia lamblia	*G. intestinalis; G. enterica*	Man
Herpetomonas ctenocephali	*Leptomonas ctenocephali*	Dog flea (may be identical with forms in other fleas)
H. muscarum	*H. muscæ-domesticæ*	Various muscoid flies
H. culicidarum	These species are taken, without considering their possible relation to other species from the same hosts, from Noguchi (1926).	*Anopheles quadrimaculatus; Culex pipiens*
H. lygæorum		Milkweed (*Asclepias*); insect (*Lygæus*)
H. media		Fly, *Calliphora*
H. muscidarum		House fly
H. oncopelti		Milkweed (*Asclepias*); insects which feed on it (*Oncopeltus* and *Lygæus*)
H. parva		Fly, *Calliphora*
Leishmania brasiliensis	*L. americana; L. peruviana.* May be identical with or a strain of *L. tropica*	Man (espundia).
L. donovani	*L. infantum*	Man (kala-azar); dog
L. tropica	*Helcosoma tropica; Herpetomonas papatasii* (?)	Man (tropical sore)
Trichomonas hominis	"Cercomonas"	Man
Trypanosoma blanchardi		Dormouse
T. brucei	*Trypanosoma pecaudi; T. togolense; T. suis*	Wild game; domestic animals (nagana)
T. congolense	*T. dimorphon* (pro parte); *T. pecorum; T. nanum; T. ruandæ*	Wild game; cattle
T. cruzi	*Schizotrypanum cruzi, T. escomeli*	Man (Chagas' disease)
T. duttoni		Mouse

II. Flagellates—(*Continued*)

Name used in present volume	*Common synonyms*	*Common hosts*
T. equinum	This together with other South American forms (*T. hippicum* and *T. venezuelense*) may be strains of *T. evansi*	Equines (mal de caderas), cattle and sheep show mild infections.
T. equiperdum		Equines (dourine)
T. evansi	*T. berberum, T. soudanense* *T. marocanum*	Equines; cattle; camels; elephants; etc. (surra)
T. gambiense	*T. ugandense; T. hominis*	Man (Gambian sleeping-sickness)
T. paddæ		Birds
T. lewisi	*T. sanguinis, T. longocaudense*	Rat
T. rabinowitschi		Hamster
T. rhodesiense	May be human strain of *T. brucei*	Man (Rhodesian sleeping-sickness)
T. rotatorium		Frog
T. vivax	*T. cazalboui, T. angolense*	Wild game; cattle; sheep; goat

III. Sporozoa

Name used in present volume	*Common synonyms*	*Common hosts*
Anaplasma centrale *A. marginale*	} May be identical; systematic position in doubt	Cattle
Babesia bigemina	*Piroplasma bigemina*	Cattle (Texas or red-water fever)
B. bovis	*Piroplasma bovis; P. divergens; B. argentina*	Cattle
B. caballi		Horse
B. canis	*Piroplasma canis*	Dog
B. equi		Horse
B. motasi		Sheep; goat
B. mutans		Cattle
B. ovis	*Piroplasma ovis; P. hirci*	Sheep; goat

III. Sporozoa—(*Continued*)

Name used in present volume	*Common synonyms*	*Common hosts*
B. sergenti	*Gonderia ovis*	Sheep; goat
Eimeria perforans	Often placed in genus *"Coccidium"*	Rabbit
E. stiedæ		Rabbit
Hæmoproteus columbæ	Often placed in genus *Halteridium*	Pigeon
Hepatozoon canis	*Leucocytozoon canis* *Hæmogregarina rotundata*	Dog
H. funambuli		Indian palm squirrel
H. muris	*H. perniciosum*	Rat
Isospora bigemina	Often placed in genus *"Coccidium"*	Dog; cat
I. felis		Dog; cat
I. revolta		Dog; cat
Plasmodium cathemerium	*P. præcox* (pro parte)	Sparrow
P. falciparum	*Laverania malariæ*	Man (malignant tertian malaria)
P. inconstans		Sparrow
P. kochi		Monkeys of Africa
P. malariæ		Man (quartan malaria)
P. præcox	*Proteosoma* or *Hæmamæba relicta*	Sparrow; small birds
P. vivax		Man (benign tertian malaria)
Sarcocystis aucheniæ	Systematic position in doubt; may all belong to one species, *S. miescheriana*	Llama
S. blanchardi		Buffalo
S. tenella		Sheep
Theileria annulata	May be race of *T. parva*	Cattle
T. dispar	May be race of *T. parva*	Cattle
T. hirci		Sheep; goat
T. parva	*Piroplasma parvum*	Cattle (East coast fever)

IV. Trematodes (Flukes)

Name used in present volume	*Common synonyms*	*Common hosts*
Clonorchis sinensis	*Distomum sinense*	Man and other mammals
Fasciola hepatica	*Distomum hepatica, F. humana*	Sheep and herbivorous animals; occasionally man
Nanophyetus salmincola	*Nanophyes salmincola*	Dog; wild carnivores
Paragonimus westermani	*P. ringeri* (?)	Man and other mammals
Schistosoma bovis	Often placed in genus *Bilharzia*	Sheep; cattle
S. hæmatobium		Man
S. japonicum		Man and other mammals
S. mansoni		Man
S. spindalis		*Bos indicus;* ruminants
Schistosomatium pathlocopticum		Natural host unknown; experimentally, rat and mouse

V. Cestodes (Tapeworms)

Name used in present volume	*Common synonyms (See also names of larval forms)*	*Common hosts*	
		Definitive hosts	*Larval hosts*
Anoplocephala perfoliata		Horse	
A. plicata		Horse	
Diphyllobothrium latum	*Dibothriocephalus latus; Bothriocephalus latissimus;* "Broad or fish tapeworm"	Man	First larva (procercoid) in crustacea, second larva (plerocercoid) in various fish
Dipylidium caninum	*Tænia cucumerina; Microtænia canina*	Dog; cat; occasionally man	Larva in fleas and lice
Echinococcus granulosus	*Tænia echinococcus; Echinococcifer echinococcus; Echinococcus multilocularis* (?)	Dog; other canines	Echinococcus or hydatid cyst of sheep, man, many mammals
Hymenolepis fraterna	May be identical with or a race of *H. nana; Tænia murina*	Rodents	Direct development

V. Cestodes (Tapeworms)—(*Continued*)

Name used in present volume	*Common synonyms (See also names of larval forms)*	*Common hosts*	
		Definitive hosts	*Larval hosts*
H. nana		Man	Direct development
Moniezia expansa		Sheep; goat; ox	
Multiceps multiceps	*Tænia cœnurus*	Dog	*Cœnurus cerebralis* of sheep, goat, ox, etc.
M. serialis	*Tænia serialis*	Dog	*Cœnurus serialis* of rabbit
Tænia crassicollis		Cat	*Cysticercus fasciolaris* of rodents
T. hydatigena	*Tænia marginata*	Dog	*Cysticercus tenuicollis* of ruminants, pig
T. saginata	*T. mediocanellata*	Man	*Cysticercus bovis* of ox
T. serrata		Dog	*Cysticercus pisiformis* of rabbit
T. solium		Man	*Cysticercus cellulosæ* of pig; occasionally in man, other mammals
Thysanosoma actinioides		Sheep	

VI. Nematodes (Roundworms)

Name used in present volume	*Common synonyms*	*Common hosts*
Ancylostoma braziliense	*A. ceylanicum*	Man; dog; cat
A. caninum		Canines
A. duodenale	*Ankylostoma* or *Uncinaria duodenale*	Man
Ascaridia columbæ	*Heterakis maculosa*	Pigeon
A. galli	*Ascaridia perspicillum*, when referring to European species	Domestic fowl
A. lineata	*A. perspicillum* when referring to American species	Domestic fowl
Ascaris columnaris		Skunk
A. lumbricoides		Man; pig; monkey

VI. Nematodes (Roundworms)—(*Continued*)

Name used in present volume	*Common synonyms*	*Common hosts*
A. vitulorum		Cattle
Bunostomum phlebotomum	*Strongylus radiatus*	Cattle; sheep
B. trigonocephalum	*Strongylus trigonocephalus, Sclerostoma hypostomum*	Sheep; goat; ox
Dioctophyme renale	*Ascaris renalis, A. visceralis, Strongylus* or *Eustrongylus gigas*	Mammals
Dictyocaulus filaria	*Strongylus filaria*	Sheep; goat
Dirofilaria immitis	*Filaria immitis*	Dog
Dracunculus medinensis	*Filaria medinensis*	Man; other mammals
Enterobius vermicularis	*Ascaris* or *Oxyuris* or *Fusarella vermicularis*	Man; monkey
Gongylonema scutatum	*Myzomimus scutatus.* May be same as *G. pulchrum* of pig	Horse; ox; goat; sheep
Graphidium strigosum	*Strongylus strigosus, S. retortæformis, S. blasii, Spiroptera leporum*	Rabbit
Hæmonchus contortus	*Strongylus contortus*	Sheep; goat
Necator americanus	*Uncinaria americana*	Man
Œsophagostomum columbianum	*Hypostomum columbiana*	Sheep; goat
O. dentatum	*O. subulatum*	Pig
Oxyuris equi	*Trichocephalus equi; Oxyuris curvula*	Equines
Parascaris equorum	*Ascaris equi; A. equorum, A. megalocephala*	Equines
Passalurus ambiguus	*Oxyuris ambiguua*	Rabbit
Stephanurus dentatus	*Sclerostomum pinguicola; S. nattereri; S. renium*	Pig
Strongyloides fülleborni		*Anthropopithecus*
S. stercoralis	*Anguillula stercoralis*	Man
Strongylus edentatus		Equines
S. equinus	*Sclerostomum equinum; S. armatus*	Equines

VI. Nematodes (Roundworms)—(*Continued*)

Name used in present volume	*Common synonyms*	*Common hosts*
S. vulgaris	*Sclerostomum vulgare*	Equines
Syngamus trachea	*S. trachealis; S. pictus; S. bifurcatus; S. mucronatus; S. primitivus; S. sclerostomum*	Turkey; chicken; birds
Toxascaris leonina	*Toxascaris marginata; Ascaris leonina; A. cati; A. limbata*	Carnivora
Toxocara canis	*Ascaris marginata; A. canis; Belascaris marginata*	Dog
Trichostrongylus retortæformis	*Strongylus retortæformis*	Rabbit
Trichinella spiralis	*Trichina spiralis*	Mammals
Trichuris trichiura	*Ascaris trichiura; Trichocephalus hominis; T. dispar*	Man
T. vulpis	*Trichuris* or *Trichocephalus depressiusculus*	Dog, fox

VII. Acanthocephala (Thorn-headed Worms)

Name used in present volume	*Common synonyms*	*Common hosts*
Macrocanthorhynchus hirudinaceus	*Echinorhynchus* or *Gigantorhynchus gigas*	Pig; man (Larvæ in insects)

VIII. Ectoparasitic Insects

Name used in present volume	*Common synonyms*	*Common hosts*
Hippobosca equina	"Horse tick"	Horse
Melophagus ovinus	"Sheep tick"	Sheep
Pediculus corporis	*Pediculus vestimenti;* "Body louse"	Man
P. humanus	*Pediculus capitis;* "Head louse"	Man
Phthirus pubis	*Pediculus pubis; P. inguinalis;* "Crablouse"	Man
Pulex irritans	*Pulex hominis; P. vulgaris*	Man; domestic animals
Spilopsyllus cuniculi		Rabbit
Xenopsylla cheopis	*Pulex cheopis*	Rat; other animals

IX. Myiasis-producing Flies

Name used in present volume	*Common synonyms*	*Common hosts*
Cordylobia anthropophaga	*Ochromyia anthropophaga;* "Tumbu fly"	Man; various mammals
Gastrophilus hæmorrhoidalis	"Red-tailed bot-fly"	Equines
G. intestinalis	*Œstrus equi*	Equines
Hypoderma bovis	*Œstrus bovis; O. subcutaneus;* "Ox-bot fly"	Cattle
H. lineatum	"Ox-warble"	Cattle
Œstrus ovis	"Sheep-bot"	Sheep

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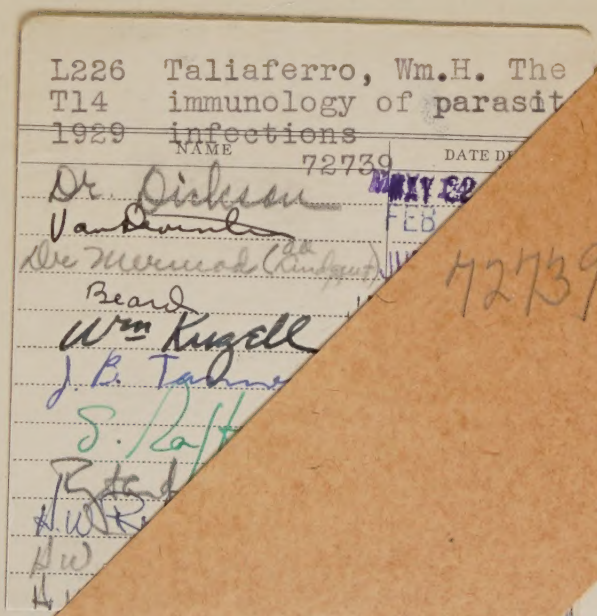
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